Comparative analysis of ACTH and corticosterone sampling methods in rats

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Vahl, Torsten P., Yvonne M. Ulrich-Lai, Michelle M. Ostrander, C. Mark Dolgas, Eileen E. Elfers, Randy J. Seeley, David A. D’Alessio, and James P. Herman. Comparative analysis of ACTH and corticosterone sampling methods in rats. Am J Physiol Endocrinol Metab 289: E823–E828, 2005. First published June 14, 2005; doi:10.1152/ajpendo.00122.2005.—A frequently debated question for studies involving the measurement of stress hormones in rodents is the optimal method for collecting blood with minimal stress to the animal. Some investigators prefer the implantation of indwelling catheters to allow for frequent sampling. Others argue that the implantation of a catheter creates a chronic stress to the animal that confounds stress hormone measures and therefore rely on tail vein sampling. Moreover, some investigators measure hormones in trunk blood samples obtained after anesthesia, a practice that may itself raise hormone levels. To address these concerns, we 1) compared plasma ACTH and corticosterone (Cort) concentrations in pre- and poststress rat blood samples obtained via previously implanted venous catheters, tail vein nicks, or clipping the tip off the tail and 2) compared plasma ACTH and Cort in rat blood samples obtained by decapitation with and without anesthesia. Rats sampled via indwelling catheters displayed lower prestress ACTH levels than those sampled by tail vein nick if the time to acquire samples was not limited; however, elevated basal ACTH was not observed in samples obtained by tail clip or tail nick when the samples were obtained within 3 min. Baseline Cort levels were similar in all groups. After restraint stress, the profile of the plasma ACTH and Cort responses was not affected by sampling method. Decapitation with prior administration of CO2 or pentobarbital sodium increased plasma ACTH levels ~13- and 2-fold, respectively, when compared with decapitation without anesthesia. These data indicate that tail vein nicking, tail clipping, or indwelling venous catheters can be used for obtaining plasma for ACTH and Cort during acute stress studies without confounding the measurements. However, the elevation in basal ACTH seen in the tail vein nick group at baseline suggests that sampling needs to be completed rapidly (<3 min) to avoid the initiation of the pituitary stress response. Death by CO2 and pentobarbital sodium injection before trunk blood collection cause significant stress to animals, as reflected in the elevated plasma ACTH levels. These results support the use of either chronic vascular cannulas or sampling from a tail vein. However, collection of blood under pentobarbital sodium or CO2 anesthesia is likely to confound the results of stress studies when ACTH is an important endpoint.

Some of the disparity might be attributable to the methods of blood collection. At present, investigators conducting stress studies use a number of techniques to collect blood, including indwelling venous catheters, tail nicks, and tail clips. In general, different laboratories have developed their own methods by trial and error, and there are not many well-controlled comparisons of the various blood-drawing techniques on plasma levels of stress hormones during short-term stress experiments.

Generally, investigators choose the sampling method on the basis of convenience or specific requirements of their study. One commonly accepted method for blood sampling in rats is the implantation of indwelling catheters (22, 23, 25, 29). Venous catheters have the advantage of simplifying repeated sampling, especially when large blood volumes are needed. While removal of 3 ml of blood or more from rats in a short period of time increases plasma Cort levels (29), the availability of a venous catheter allows for either the transfusion of donor blood (30) or the return of erythrocytes suspended in sterile saline such that hypovolemia and anemia can be prevented during serial sampling.

However, there are several real and potential problems raised by the use of long-term intravenous catheters in stress studies. Placement of indwelling catheters requires surgical skill and is invasive to the animal. There is a legitimate concern that the implantation and presence of a catheter is itself a source of chronic irritation (25). If indeed the catheter represents a chronic stressor, one could expect to observe facilitation of hypothalamic-pituitary-adrenal (HPA) axis stress responses in the wake of catheter implantation. Indwelling venous lines can cause other severe consequences as well. For example, central venous catheters inserted under sterile conditions in patients in U.S. hospitals lead to infections and thrombotic complications in up to 26% of cases (15, 19). Similar to humans, placement of indwelling venous catheters can be associated with the development of septic thrombophlebitis and generalized sepsis in rodents, complications that can be avoided to some extent by sterile surgical technique (18). If an infection is not recognized, the outcome of a stress study could be easily confounded. While some suggest filling the catheters with a solution that contains antibiotics during the time that they are not being used (23, 24), this might not be suitable for certain studies unless the experimenter can be sure that there are no interactions between the antibiotic and the dependent variable of interest.

A commonly used alternative is blood collection from a tail vein (7, 10, 12, 17, 27, 31). This method has a different set of
advantages and drawbacks. Tail vein nicking and tail clipping do not require surgery and are therefore simpler, less invasive procedures. For this reason, many investigators have favored these techniques in stress studies. However, depending on the specific technique, animals may need to be lightly and temporarily restrained at each sampling point, which could represent an additional stressor ("restressing") that can alter secretory patterns of circulating stress hormones. Thus there is currently no single method that has been generally accepted as the standard for collecting blood during stress experiments. While experienced investigators and laboratories may have techniques that have proven successful, the lack of a methodological standard confounds comparisons among studies and can be confusing to investigators new to stress research.

Another source of debate in the conduct of stress studies is the best method for blood collection at the time of death. The recommendation of the American Veterinary Medicine Association (AVMA) panel on euthanasia suggests the use of decapitation, in cases where there is scientific justification and approval by the Institutional Animal Care and Use Committee, to allow for the collection of tissues and body fluids free of chemical contamination (1, 2). Frequently, animals are exposed to CO2 or given an injectable anesthetic before decapitation. However, investigators interested in the measurement of stress hormones have argued that CO2 exposure or injection of anesthetics can delay blood collection and elevate HPA axis hormones independently, confounding the experimental results.

To address the question of sampling method on levels of stress hormones, the current study compared the plasma ACTH and Cort levels in blood samples obtained from implanted central venous catheters, tail vein nick, and tail clip. In addition, we compared decapitation after anesthesia (CO2 or pentobarbital sodium) with unanesthetized decapitation.

MATERIAL AND METHODS

Subjects. Subjects were male Sprague-Dawley rats weighing between 300 and 350 g (Charles River Laboratories, Wilmington, MA). All rats were housed singly and maintained on a 0700–1900 light-dark cycle with free access to standard chow and water. All animal procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Experiment 1. Two groups of rats (n = 12/group) were used in this experiment; one group had vena cava catheters implanted for blood sampling, and the other group underwent no surgery and blood was sampled from the tail vein as indicated below.

The vena cava catheters were implanted under pentobarbital sodium anesthesia (45–60 mg/kg). The abdomen was opened by a midline incision, and the intestine was carefully moved aside such that the inferior vena cava was visible. The inferior vena cava was punctured with a 21-gauge needle just below the junction with the abdominal incision, and the Silastic catheter (0.025 ID x 0.018 OD; Technical Products) was inserted 3 cm in the vein with the tip being placed just outside the right atrium. The lines were sutured in place, threaded through a puncture in the wall of the abdomen, and tunneled subcutaneously to the top of the skull where they were connected to sampling ports and secured with dental cement. All rats were allowed to recover from surgery for at least 1 wk and had regained their preoperative body weight before the study. Venous catheters were checked 48 h after surgery and filled with heparinized saline (100 U/ml) to maintain patency. On the day of the experiment, the heparinized saline was removed immediately before the first blood sample was taken, and lines were flushed with normal saline during the experiments. To obtain blood from the vena cava catheters, rats were briefly wrapped in a towel, and polyethylene tubing was connected to the sampling ports for each blood draw. The catheters were flushed with 500 μl normal saline after each sample and closed with a polyethylene cap.

As mentioned above, a second group of rats underwent no surgeries and had blood sampled from the tail. On the days leading up to the study, this second group was handled similarly to the vena cava group when these animals had their catheters checked for patency. During the stress experiments, tail samples were collected from a small vertical incision along the side of the tail by milking blood from a lateral tail vein into nonheparinized capillary tubes while the rats were restrained. The blood was then filled in microcentrifuge tubes containing EDTA and placed on ice. In our experience, it is important to milk the tail gently to avoid damaging the skin, especially during serial sampling. It can help to wear latex or Nitrile gloves that are turned inside out since the inside of the glove is extremely smooth and slides easily along the skin with minimal abrasion. High manual pressure during the milking of the tail can also cause hemolysis, which may interfere with the accuracy of the measurements obtained by RIA.

On the day of the study, the animals were individually transported (by a person that was dedicated to the transport of the animals) to a nearby test room where a baseline blood sample was taken immediately. The baseline blood samples were obtained between 0830–0930, and a total of 12 rats were tested in one morning. Two rats were studied in parallel (one from each group) by two investigators such that blood was collected from a rat with a vena cava catheter and from a rat for tail blood sampling at the same time. The animals were placed in well-ventilated polyethylene tube restrainers for 30 min to induce a stress response. As soon as the first animal had its blood sample taken and its 30-min restraint period had started, the next animal was brought in the test room for the collection of the baseline sample. Additional blood samples were taken at 30 min (the end of the restraint) and at 60 and 120 min. For tail vein nicks, rats were placed back in the restraining tubes, and incisions were made at progressively proximal points on the tail at each sampling point. Typically, it required 3–4 min after the initial nick to obtain sufficient blood (200–300 μl) for analysis; subsequent nicks bled more quickly (1–3 min).

Experiment 2. One of the problems associated with the tail vein nick procedure is the time needed to collect sufficient blood for the ACTH assay. The time interval from cage removal to completion of sampling can approach 5 min in some cases, during which plasma ACTH levels can rise. Accordingly, this second experiment was designed to test the relationship between time taken to collect samples and basal plasma ACTH levels. Animals were placed in plastic restraint tubes between the hours of 0900 and 1000, the tail veins were immediately incised with a stitch scalpel, and 200–300 μl of blood were collected in tubes containing EDTA. The time from incision until completion of the sample was measured on a standard laboratory timer.

Experiment 3. Pain is another potential confound to the collection of blood for measurement of stress hormones. To test this possibility, we compared tail nicks with an alternative tail sampling procedure, tail clips, that allows for faster sampling but potentially different levels of pain and stress. For tail clips, the distal 0.5 mm of the tail is turned inside out since the inside of the glove is extremely smooth and slides easily along the skin with minimal abrasion. High manual pressure during the milking of the tail can also cause hemolysis, which may interfere with the accuracy of the measurements obtained by RIA.

To do this, the tip of the tail was gently wiped with a piece of sterile gauze. For this study, groups of animals were removed from their home cage between the hours of 0900 and 1000 and placed in restrainers for 30 min. Tail blood (200–300 μl) was sampled by either tail vein nick (n = 6) or tail clip (n = 6) at 0, 30, and 90 min after the onset of restraint.
Experiment 4. This experiment was carried out between 0830 and 0930. Trunk blood was collected from three different groups of rats after decapitation following: 1) death by CO₂, 2) pentobarbital sodium injection, or 3) no anesthesia. All animals were individually transported in their home cage to the necropsy room (transport time ~20–30 s). Rats euthanized in the CO₂ chamber (JorVet J-265; Jørgensen Laboratories, Loveland, CO) were exposed to the gas until they stopped breathing (~2 min). Pentobarbital sodium was given as an intraperitoneal injection of 150 mg/kg pentobarbital sodium (Sigma, St. Louis, MO), and rats were decapitated when they reached a surgical anesthesia plane (~3–5 min). Animals sampled without anesthesia were removed from their cages and decapitated within 10 s.

Plasma analysis. Blood for hormone measurements was collected in either EDTA-Vacutainer tubes (for 3 ml trunk blood) or in Eppendorf tubes containing 20 μl of 0.05 M EDTA (for 500 μl tail and catheter samples). Blood was immediately placed on ice and centrifuged, and plasma was stored at −20°C until measurement. ACTH was measured using a RIA kit (DiaSorin, Minneapolis, MN) according to the manufacturer’s instructions; this assay requires 50 μl plasma/RIA tube. The limit of detection for this assay was 15 pg/ml, and intra- and interassay coefficients were 6.3 and 6.0%, respectively. Cort was also measured by RIA (ICN, Costa Mesa, CA) from a 10-μl sample that was diluted 1:20 (basal) or 1:200 (poststress). The Cort RIA kit has intra- and interassay coefficients of variation of 7.1 and 7.2%.

Statistical analysis. Results are given as means ± SE. Two-way ANOVA for repeated measures was used for comparison of groups over time, and a one-way ANOVA was used to detect differences in experiment 4. When necessary, homogeneity of variance was attained by performing analyses on the values after log transformation. Specific differences were determined by Scheffé’s post hoc analysis. Student’s t-test was used for comparison of baseline values in experiment 1. Differences were considered to be significant for P values <0.05.

RESULTS

Experiment 1. Plasma ACTH values before and after restraint stress are shown in Fig. 1, top. Basal ACTH concentrations were greater in the tail nick group than in the venous catheter group (154.5 ± 15.8 and 62.8 ± 6.4 pg/ml, respectively, P < 0.01), possibly reflecting the longer time period required to obtain the sample from the tail vein. However, both groups of animals showed a similar response to stress, with peak values that did not differ significantly (220.0 ± 20.1 and 178.1 ± 21.2 pg/ml for the tail nick and venous catheter groups, respectively) and a return of ACTH toward basal by 120 min.

Plasma levels of Cort before and after restraint stress are shown in Fig. 1, bottom. Baseline Cort values were greater in the tail nick group than in the catheter group (34.0 ± 7.9 and 8.8 ± 3.2 ng/ml, respectively, P < 0.01; values that were consistent with the differences observed in ACTH). After restraint, levels of Cort peaked at 30 min and did not differ between groups (414.3 ± 45.4 ng/ml for the catheter-implanted rats and 470.3 ± 37.0 ng/ml for the tail vein nick group). Thereafter, plasma levels of Cort returned toward basal levels and also did not differ between groups.

Experiment 2. Plasma ACTH in animals sampled by tail nick was correlated with the time required to collect the sample (r = 0.61, P = 0.002; Fig. 2). Samples collected over the interval of 100–180 s had an average value of 51.6 ± 6.6 pg/ml, consistent with values obtained upon initial sampling in animals with catheter implants (see above). However, once the sampling period exceeded 180 s, ACTH values began to rise substantially. From these data, it appears that blood sampled from the tail vein needs to be collected in <3 min to achieve true basal values.

Experiment 3. The tail clip sampling procedure is somewhat different from tail vein nick, since it involves a different cut procedure (removal of the distal tip of the tail vs. lateral incision) and a different number of cuts (1 vs. ≤4). Because these differences could affect stress hormone responses, we compared ACTH and Cort before and after restraint stress using the clip and nick methods. In this experiment, blood collection time was capped at 3 min in both groups. As depicted in Fig. 3, the two tail sampling procedures produced similar stress hormone profiles, with no significant differences in basal levels, peak responses, or return to baseline.

Experiment 4. In the last experiment, plasma ACTH levels for animals decapitated with prior CO₂ exposure were ~13-fold higher, and those from rats given a lethal injection of pentobarbital ~2-fold higher, than nonanesthetized rats (596.1 ± 97.7, 131.5 ± 30.0, and 43.1 ± 5.4 pg/ml, respectively, P < 0.001; Fig. 4). Notably, the ACTH concentrations in the nonanesthetized trunk blood measurements were very similar to the baseline values we found in the rats implanted with venous catheters or with the tail clips (43.1 ± 5.4 vs.
62.8 ± 6.4 vs. 16 ± 7.4 pg/ml, respectively). CO2 exposure and pentobarbital sodium injection before decapitation also led to an elevation of plasma Cort levels (56.4 ± 33.5 and 83.3 ± 46.1, respectively, compared with 9.3 ± 3.8 ng/ml, \( P < 0.05 \); Fig. 4). Interestingly, the plasma Cort concentration was significantly higher after pentobarbital sodium injection compared with the nonanesthetized group (\( P < 0.05 \)), despite relatively lower plasma ACTH concentrations.

**DISCUSSION**

In this study, we sought to determine the relative effects of different methods of blood sampling on the response of the HPA axis in rats. Our results indicate that blood sampling from either venous catheters or tail veins can be used in stress studies without confounding the outcome. However, both methods have strengths and weaknesses, and the optimal method should therefore be chosen carefully according to the experimental protocol. Baseline blood samples should be collected in 2–3 min so that sampling is completed before activation of the HPA axis. For collection of trunk blood in terminal studies where plasma ACTH and Cort are important measures, prior CO2 administration and pentobarbital sodium anesthesia should be avoided.

It has been previously reported that indwelling central venous catheters in rats cause a local inflammatory response at the fixation site and small emboli in the lungs (6). These effects and pain or discomfort caused by the catheter are potential stressors that could confound stress studies. Rats that have been exposed to chronic stressors react to novel stress exposures with a facilitated plasma ACTH and Cort response (3).
This phenomenon could affect the stress hormone response in animals that underwent catheter surgery, and to our knowledge this question has not been tested systematically. In our experiments, we did not find any difference in the plasma ACTH and Cort response profiles between rats with and without catheter implantation. This suggests that carefully implanted catheters do not cause facilitation of HPA axis responses to novel stressors. Catheters are particularly useful in experiments requiring large samples and short time intervals, or under conditions when blood samples must be obtained from unhandled, undisturbed subjects (e.g., remote sampling). In this study, the rats tolerated vena cava catheters for a period of 1 wk without any apparent disruption of their normal behavior. Earlier studies have reported that venous catheters do not affect plasma Cort concentrations (25, 26). Plasma Cort levels are increased immediately after surgery (20) but usually return to normal within 2–5 days (25). The surgery also leads to a transient reduction in food intake and body weight followed by normal weight gain (4, 18). Our studies confirmed that baseline plasma Cort levels 1 wk after the surgery are not elevated and extend this finding to plasma ACTH as well. Samples taken from catheterized animals before stress were consistent with basal ACTH values from the literature (<50 pg/ml in studies utilizing the same RIA; see Ref. 5). Therefore, the use of intravenous catheters is a reasonable option for blood sampling in stress research. However, catheter implants are labor intensive for studies where only one or two blood samples have to be taken. In addition, with longitudinal experiments over several weeks or months, some catheter failures will inevitably occur, necessitating preparation of extra subjects. Catheters are also contraindicated under experimental conditions where group housing is mandated, or interindividual behaviors are measured, since catheters can be damaged by interactions with conspecifics.

Collection of blood from the tail of rodents is a technically less demanding option that can be learned very quickly. However, serial sampling from tail veins caused a moderate but significant increase in plasma Cort levels in some studies (8, 12). This does not seem surprising given that movement of the cage or opening of the cage lid (9) can initiate a hormonal response similar to that of restraint (5, 10) and exposure to a novel environment (7), all of which are partially involved in obtaining tail blood samples. In another study, sampling from tail incisions was shown to have only a minor effect on plasma Cort even during repeated collections over 3 h (7). These investigators found no significant increase in baseline Cort secretion in rats that were well handled during the days before the tail blood sampling and that remained in their home cages between the individual blood samples, whereas rats that were placed in a novel cage showed a strong stress response. Moreover, simultaneous sampling from an intravenous catheter and from the tail after subcutaneous Cort injections resulted in equal plasma Cort concentrations (7).

We believe that differences in the sampling time account for the difference in baseline ACTH and Cort results between the tail nick and catheter groups in experiment 1. In this study, there were significantly greater plasma ACTH and Cort levels before restraint stress in the tail nick animals compared with catheterized rats. In this experiment, we did not emphasize the time of collection during the tail nick sampling, which was obtained over a longer period, 3–5 min. After collecting these results, we considered the importance of timing as a factor in the tail nicking procedure in experiment 2. Here we found a clear correlation between sampling time and ACTH. When samples were collected in <3 min, ACTH levels were consistent with basal values (51.6 pg/ml; see Refs. 8 and 10), whereas levels began to rise precipitously if sampling time was extended. These results are similar to those of Liu et al. (14) in which plasma Cort sampled by arterial catheters correlated with time to acquire the sample. Thus venous cannulation and tail nick/tail clip sampling both constitute appropriate approaches, with the proviso that the sampling time is held to <3 min.

In terminal experiments, collection of trunk blood after decapitation is commonly used. The use of decapitation for collection of tissues and blood samples is a controversial issue (see report of the AVMA panel on euthanasia 1993 and 2000 in Refs. 1 and 2, respectively). Concerns about unanesthetized decapitation protocols were raised in a study by Mikeska and Klemm (16) indicating that electrical activity persists in the brain for 13–14 s after decapitation. Although the authors interpreted these findings as a sign of pain perception, others have demonstrated that the measured electroencephalogram activation is also seen under inhalation anesthetics and during rapid-eye-movement sleep (reviewed in Ref. 13). Despite the controversy, the AVMA recommends that rodents be anesthetized or asphyxiated with CO$_2$ before decapitation, unless adequate scientific justification is provided. Based on the levels of plasma ACTH and Cort, our study suggests that animals anesthetized or euthanized before decapitation exhibit substantial stress responses, probably because of a combination of additional handling, exposure to anesthetic, and the time required for the rats to lose consciousness. In contrast, rats euthanized by rapid conscious decapitation had plasma ACTH values that did not differ from the normal basal levels that we saw in either cannulated or tail-sampled animals before restraint. These findings are consistent with previous work suggesting that this procedure causes a rapid cessation of neural activity without antecedent stress (13, 28). Together, the scientific evidence indicates that rapid decapitation is a humane method for stress-free euthanasia of rats.

In conclusion, our studies indicate that both catheterization and tail vein sampling are equally useful methods for sampling stress hormones in rats, if performed carefully by efficient personnel. This conclusion is drawn from the observation that blood sampling from indwelling venous catheters and via tail nick/clip yields normal basal levels of plasma Cort and ACTH, and identical stress profiles. Thus it is unnecessary to incorporate catheterization into all stress hormone measurement protocols. Conversely, in protocols that require catheters, the procedure does not necessarily alter basal HPA axis tone or HPA axis stress responses, but sterile implantation techniques should be used. The choice of sampling protocol can thus be selected on the basis of design requirements, without fear that either method will result in erroneous results. However, it is clear that, for tail sampling, speed is of the essence, and it is important to keep the sampling time limited to 2–3 min. For terminal measures, anesthesia and CO$_2$ administration before decapitation elevate plasma stress hormones, and the use of such procedures in designs requiring rapid hormone or tissue sampling is problematic.
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