Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 381-385. Pergamon Press. Printed in Great Britain

EVALUATION OF ADRENAL FUNCTION IN MICE BY MEASUREMENT OF URINARY EXCRETION OF FREE CORTICOIDS

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(*Received* 8 *September* 1975)

SUMMARY

Methods for the determination of urinary total free corticoids and urinary free corticosterone in small laboratory rodents are described. Total free corticoids and free corticosterone were determined in samples of 50–200 μl urine by a competitive protein binding method using human plasma transcortin as the source of binding protein. For both methods employed the reliability criteria were satisfactory. The validity of both methods was tested by measuring both total free corticoids and free corticosterone in urine samples of mice with the obese-hyperglycemic syndrome (gene symbol C57BL/6J-ob/ob) and their lean controls (gene symbol C57Bl/6J). Measurements were taken from urine samples under basal conditions and after adrenal stimulation or suppression. Urinary corticoid levels were significantly different between the two mouse lines, 15 ng/day for the lean and 75 ng/day for the obese mice, reflecting plasma corticosterone levels. After stimulation by ACTH urinary corticoids rose 3–5-fold whereas suppression by dexamethasone was followed by a decrease to very low levels. The contribution of corticosterone to total corticoids averaged 50% in lean and 30% in obese mice. It is concluded that urinary total excretion of free corticoids as well as urinary excretion of free corticosterone offer a valuable parameter of adrenal function in mice. Furthermore, when small laboratory rodents are used, under many circumstances measurements in urine are more advantageous than measurements in plasma.

INTRODUCTION

Assessment of adrenocortical function in humans and rodents is usually obtained measuring plasma cortisol or plasma corticosterone levels, respectively. However, plasma corticosterone concentrations in small laboratory rodents display considerable diurnal variation [1]. Furthermore, plasma corticosterone levels are influenced by stress [2], which is shown by the marked effect of different procedures of sacrifice [3]. It is even impossible to avoid stress-induced corticosterone secretion when an adrenal function test is to be performed which requires repeated samplings of blood. These methodological difficulties led us to investigate the posssibility whether adrenocortical function in small laboratory rodents can be assessed by analysis of urinary corticoid excretion.

We used two inbred lines of one strain, namely C57BL/6J-ob/ob mice and C57BL/6J mice in which the ob gene is lacking. Since basal plasma corticosterone levels in C57BL/6J-ob/ob mice are significantly higher than in C57BL/6J mice, indicating a hyperadrenocorticoidism [4, 5], these animals are suitable models for testing whether adrenal function is reflected by urinary excretion of free corticoids. Free corticosterone and total free corticoids in the urine of mice were assayed under basal conditions and after stimulation by ACTH and suppression by dexamethasone.

Supported by the Deutsche Forschungsgemeinschaft (Kl 346). Request for reprints should be addressed to: Priv. Doz. Dr. H. K. Kley, 2. Medizin. Universitätsklinik, 4 Düsseldorf, Moorenstr. 5, Germany.

MATERIALS AND METHODS

Chemicals. Solvents and reagents (analytical reagent grade) were purchased from Merck, Darmstadt, Germany; Dextran 80 and Sephadex LH-20 from Pharmacia Uppsala, Sweden. [1,2-^{3}H]-corticosterone (S.A. 50 Ci/mmol) was obtained from the New England Nuclear Chemicals GmbH, Frankfurt, Germany. On arrival it was diluted with benzene-ethanol (9:1, v/v) to a concentration of 10 μCi/ml and stored at 4°C. Every three months it was repurified by column chromatography. Unlabelled corticosterone was obtained from Ikapharm, Ramat-Gan, Israel and stored at 4°C at a concentration of 10 μg/ml ethanol from which a working standard containing 0.1 μg/ml ethanol was made monthly. The transcortin-isotope solution was made as follows [6]: 1.0 ml human plasma, collected 8 h after administration of 3 mg dexamethasone or 1.0 ml plasma of mice (C57BL/6J) was dissolved in 80.0 ml phosphate buffer (p_H:7.4; 0,05 M) containing 10 000 c.p.m. labelled corticosterone/ml.

Animals. Male C57BL/6J-ob/ob mice (mean body weight 46 g) and male C57BL/6J mice (mean body weight 32 g) about 4 months of age were used. The animals came from brother-sister-inbred lines maintained at the Diabetes Forschungsinstitut, Düsseldorf, and originating from The Jackson Laboratories, Bar Harbor, Maine, U.S.A. The animals were housed,

10–15 to a cage (floor area 23 × 39 cm.), in a temperature-controlled room (24 ± 2°C, relative humidity 55%) with a light–dark cycle (6 a.m. until 6 p.m.) and had free access to a standard mouse chow (Intermast, Soest, Germany) and tap water.

Experimental procedure. Four days prior to the experimental period single animals were placed into metabolism cages* with free access to food and tap water. The separation of urine and feces in this cage was accomplished by allowing urine to flow along the inner surface of a separator funnel into a urine collecting bowl, while feces fell by gravity into a feces collecting bowl. During the experimental period, urine was collected in plastic tubes in daily portions and frozen at −18°C until assayed.

Time-course of urine collections: 24 h urine portions were collected under basal conditions (for 4 days), under application of ACTH (Synacthen, CIBA, Wehr, Germany), which was given intraperitoneally (0.125 U ACTH diluted in 0.5 ml saline at 8 a.m. and 6 p.m.) and after 3 days of application of dexamethasone (25 μg/ml drinking water). 0.125 U ACTH are sufficient for inducing maximal adrenal stimulation as has been tested by measurements of plasma corticosterone in control experiments.

For the exact determinations of urine volumes (vol.) we used 2 methods which led to identical results:

1. Determination of the weight of the urine sample (w) and determination of the specific weight (sw) of an aliquot (g/ml).

$$w/sw = \text{vol. (ml)}$$

2. According to the volume of the urine sample (0.2–1.0 ml) 1000–5000 c.p.m. (c.p.m. t) of labelled corticosterone was added. After equilibration the radioactivity in an aliquot (c.p.m. a/vol. a) was measured.

$$(\text{vol. a}/\text{c.p.m. a}) \times \text{c.p.m. } t = \text{vol.}$$

In addition the radioactivity added to the urine sample served as internal standard to determine sample loss during the procedure. When working with method 2, an amount of radioactivity was chosen which corresponds to 1000 c.p.m. in the volume used for determination. Method 1 is used in the following description. All samples used for radioactivity determinations were dissolved in 3.0 ml dioxane and 10.0 ml Liquiflor (New England Nuclear Chemicals) solution, thoroughly mixed and counted in a Packard Liquid Scintillation Counter (3380/544).

Measurement of free total corticoids in urine. The method employed is closely patterned after that previously described for plasma corticoids in mice [5] and for urinary corticoids in humans [8, 9]. A vol. of 0.05 to 0.2 ml of a 24 h urine sample was pipetted into a small extraction tube and the volume was made up to a total of 0.5 ml with 100 μl of an aqueous ^{3}H-corticosterone solution, containing 10,000 c.p.m./ml and the remainder with distilled water. Steroids were extracted with 8.0 ml dichloromethane. The aqueous layer was removed by aspiration and the organic phase was washed successively with 1/16 vol. of 0.1 N NaOH, 0.1% acetic acid and 3 times with distilled water. 1.0 ml was pipetted into a counting vial for the estimation of procedural loss and 2 aliquots (1/4 and 1/2 vol.) were transferred into separate centrifuge tubes and dried. 1.0 ml of the transcortin-isotope solution was added to each dried extract and to the dried standards (duplicates of zero, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 and 30.0 ng corticosterone/tube). The tubes were equilibrated at 45°C for 5 min and then cooled for 20 min in an ice bath. 0.5 ml of Dextran-coated charcoal solution (0.02% Dextran and 0.2% charcoal in saline) was added to each tube by means of an automatic pipette (Fe 213, Braun, Melsungen, Germany). After 10 min the tubes were centrifuged (3000 g) at 4°C. The supernatant was decanted into a counting vial, mixed with the scintillation solution and counted to a preset count of 10,000 c.p.m. From the measured c.p.m. of each standard the % bound was calculated and plotted against ng corticosterone to provide a standard curve (Fig. 1). The amount of corticosterone in the urine samples was read off according to the % bound fraction and corrected for procedural loss. In the standards 100% equals the radioactivity in the 1.0 ml of the transcortin-isotope solution given to each vial, whereas to the 10,000 c.p.m. in the samples the radioactivity, added to calculate procedural loss, must be summed up. These c.p.m. are measured in the recovery vials, the amount being betweeen 150–500 c.p.m. depending on procedural loss and the volume used for the assay.

Measurement of free corticosterone in urine. In order to measure free corticosterone the washed and dried urinary extracts were chromatographed over a Sephadex LH-20 column (10 × 340 mm) using dichloromethane–ethanol (99:1, v/v) [7]. The fraction with corticosterone was collected. With 1/6 of the eluate the procedural loss was determined. In aliquots of 1/2 and 1/4 vol. the amount of corticosterone was determined in the same way as for total corticoids. Using this column chromatographic separation the corticosteroids cortisol, 11-deoxycortisol, cortisone, corticosterone and 11-deoxycorticosterone are definitely separated.

RESULTS

Reliability criteria are summarized in Table 1. In order to determine precision and accuracy, pooled urine of the lean and of the obese mice were divided into several batches. Water blank values were smaller than 0.2 ng/tube. They were not subtracted from urine values.

After addition of different amounts of corticosterone (10, 20 and 40 ng/ml) to pooled urine the recoveries were 107, 102 and 89.5% by measurement with-

* Built according to a model given by the courtesy of Dr. G. Meister, Farbenfabriken Bayer, Wuppertal, Germany.

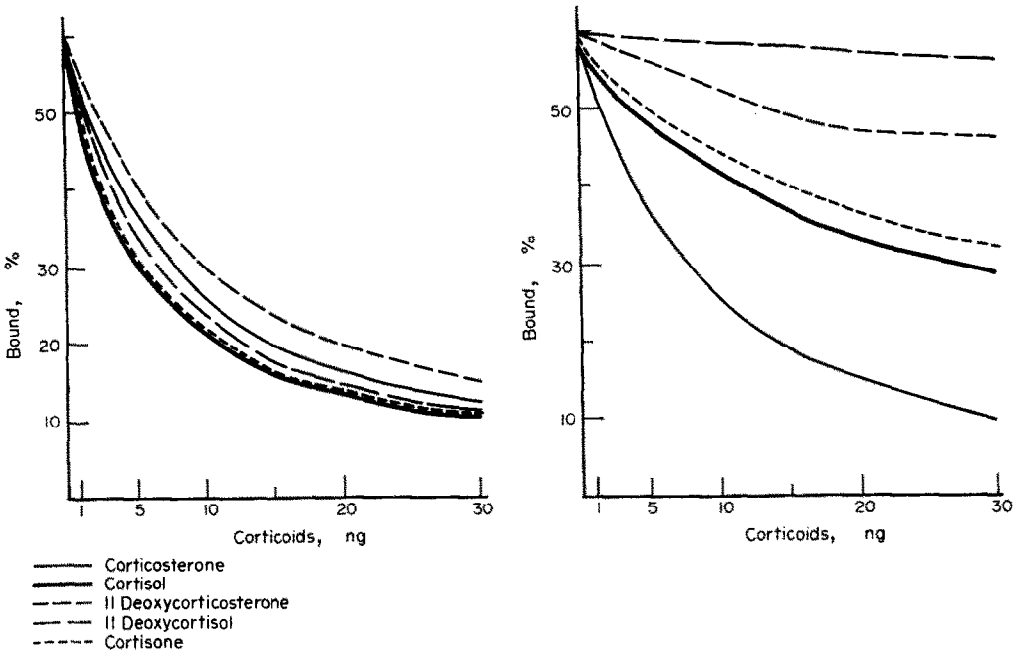

Fig. 1. Standard curves of 5 different corticosteroids using human (left side) and mouse (right side) plasma as the source of binding protein.

out chromatography and 107, 97 and 96% when free corticosterone was determined. The lowest concentration distinguishable from zero (sensitivity), calculated from duplicates of the lower range (0.5–4.9 ng/ml) was 0.3 ng/tube, i.e. 3.0 ng/ml.

When adding cortisol, 11-desoxycortisol, cortisone or 11-deoxycorticosterone to the pooled urine samples prior to chromatography, no steroids other than corticosterone were found in the eluted corticosterone fraction. As shown by the standard curves of some corticosteroids (Fig. 1) the affinity of human and mouse transcortin is different. Human transcortin binds all the 5 corticosteroids almost equally well whereas transcortin of mice (C57BL/7J) binds predominantly corticosterone and far less the other corticosteroids tested.

Table 2 shows the results from the measurement of corticoids and free corticosterone in urine of mice under basal conditions as well as after stimulation and suppression of adrenal function by ACTH or dexamethasone. Under all investigations the amount of total corticoids in urine is greater than the amount of free corticosterone. A highly significant correlation between urinary free total corticoids and urinary free corticosterone was found in both lines of mice (C57BL/6J mice: $y = 1.49 + 0.47x$; n:56; r:0.969; s_b:0.024 and C57BL/6J-ob/ob mice: $y = 10.91 + 0.30x$; n:41; r:0.836; s_b:0.036). The results show that in the lean mice 47% of the urinary free total corticoids is corticosterone whereas in the obese mice 30% of the urinary free total corticoids is corticosterone. Under basal conditions the amount

Table 1. Reliability criteria for the measurement of free total corticoids and free corticosterone in urine of mice

	Total free corticoids			Free corticosterone		
	$\bar{x} \pm$ S.D. (ng/ml)	CV (%)	n	$\bar{x} \pm$ S.D. (ng/ml)	CV (%)	n
Precision						
1. Intraassay	30.5 ± 1.9	6.1	8	16.4 ± 1.5	9.1	8
	70.2 ± 4.6	6.6	8	30.2 ± 2.5	8.1	8
2. Interassay	31.2 ± 2.6	8.2	12	16.4 ± 1.8	10.5	12
	72.1 ± 6.6	9.1	12	36.2 ± 3.5	11.2	12
Accuracy						
1. Recovery from urine						
Pool	30.5 ± 1.9	6.1	8	16.4 ± 1.5	9.1	8
Pool plus 10 ng B/ml	41.2 ± 2.9	7.1	6	27.1 ± 2.7	10.5	6
Pool plus 20 ng B/ml	50.9 ± 4.0	7.9	6	35.8 ± 3.5	9.8	6
Pool plus 40 ng B/ml	66.2 ± 5.7	8.6	6	54.8 ± 6.4	11.6	6
Recovery of internal standard	94.3 ± 2.4	3.0	80	71.7 ± 2.6	3.8	80
Sensitivity (ng/tube)	0.3			0.3		

Mean ± S.D.; CV: coefficient of variation; n: number of determinations; B: corticosterone

Table 2. Urinary total free corticoids, urinary free corticosterone and plasma corticosterone in two lines of mice: lean mice = C57BL/6J, obese mice = C57BL/6J-ob/ob

	Basal		Urine (ng/day) ACTH		Dexamethasone		Plasma corticosterone* (μg/100 ml)	
	$\bar{x} \pm$ S.D.	n	$\bar{x} \pm$ S.D.	n	$\bar{x} \pm$ S.D.	n	$\bar{x} \pm$ S.D.	n
C57Bl/6J:								
Total corticoids	15.1 ± 6.2	24**	75.1 ± 17.6	4	2.3 ± 2.0	4		
Corticosterone	7.9 ± 3.7	24**	35.2 ± 9.7	4	0.2 ± 0.2	4	1.9 ± 0.2	10
C57Bl/6J-ob/ob:								
Total corticoids	74.6 ± 11.8	24**	210.1 ± 42.3	4	7.2 ± 3.8	4	—	
Corticosterone	29.5 ± 8.3	24**	88.9 ± 18.7	4	0.4 ± 0.3	4	13.7 ± 1.5	10

* Plasma values have been published [5].
** 6 different animals, whose urinary corticoids were measured on 4 consecutive days.
Mean ± S.D.; n: number of animals

of free total corticosteroids in urine of lean mice is clearly lower than that which was measured in the obese mice, i.e. 15.1 ± 6.2 ng/day vs 74 ± 11.8 ng/day, respectively. This difference in urinary excretion of free corticoids is even more pronounced when the adrenal gland is stimulated by ACTH. The absolute increase in free corticosterone and in total free corticoids is significantly greater in the obese than in the lean mice. After application of dexamethasone a decrease of urinary free corticoids was found in all animals. In most of the animals after dexamethasone treatment the corticosterone levels were below the limits of sensitivity of the method.

DISCUSSION

Corticosterone is the major corticosteroid synthesized in the adrenals of the rat and the mouse [10, 11]. However, on evaluation of adrenal function in small laboratory rodents on the basis of measurements of plasma corticosterone some difficulties may arise: Often increased corticosterone levels are the consequence of inadequate prolonged sacrifice procedures before blood sampling. Only by a very rapid and cautious blood sampling technique can one avoid stress-induced increase in plasma corticosterone levels, as has been observed in rats [2] and mice [5].

Unspecifically increased corticosterone levels may also result when blood is removed from the tail vein or from the periorbital sinus. This is especially true when experiments are performed which involve repeated blood sampling. Repeated puncture of the periorbital sinus within the short time periods usually needed for glucose tolerance or insulin secretion tests or for adrenal function tests often renders the animals unsuitable for further experimentation. Our results show that these difficulties in evaluation of the adrenal function can be avoided by measuring the free total urinary corticoids. Because of the high correlation between urinary total free corticoids and urinary free corticosterone purification by chromatography can be omitted. If the elimination of chromatographic purification is desired the use of mouse plasma as binding protein results in an increased specificity of the method. As can be seen in Fig. 1 the affinity of mouse transcortin as the binding protein for corticosterone is much higher than that of human transcortin.

The measurement of urinary total free corticoids is not only suitable for the evaluation of adrenal function before and after administration of ACTH and dexamethasone, but may also be used for detection and investigation of inherited alterations of the adrenal gland, as is shown in genetically obese-hyperglycemic mice (Table 1).

The contribution of free corticosterone is 30–50% of the urinary total free corticoids. A similar relationship between cortisol and 11-OH-steroids has been found in human urine [12]. Previous investigations (unpublished data) show that this technique of urinary total free corticoid measurement is also suitable for other rodents, such as rats, guinea-pigs and rabbits.

In some strains of mice corticosterone as well as 11-deoxy-cortisol is synthesized by the adrenal gland [13] and thus in these strains urinary total free corticoids may better reflect adrenal function than urinary free corticosterone. However, when a separation is desirable in such strains of mice the column chromatographic separation described can be used to determine both corticosterone and 11-deoxy-cortisol in the same assay. In both lines of mice tested in this study 11-deoxycortisol was detected neither in urine nor in plasma.

On comparison of urinary excretion of total free corticoids and body weight it can be seen that humans (mean body weight 70 kg, free urinary cortisol excretion 32.5 μg/day (unpublished data)) and metabolically intact mice of the C57BL/6J line (mean body weight 32 g, free urinary total corticoids 15.1 ng/day) have practically identical corticoid/body weight ratios, i.e. 465 ng/kg/day and 472 ng/kg/day, respectively. In metabolically obese mice of the C57BL/6J-ob/ob line (mean body weight 46 g, free urinary total corticoids 74.6 ng/day), however, the ratio is 1620 ng/kg/day. As has been shown for humans the renal excretion of urinary total free corticoids is directly correlated to the concentration of non-protein bound (metabolically active) corticosteroids in plasma (for

lit. see 14). Therefore the measurement of urinary total free corticoids effectively reflects alterations in adrenal function ("lens-effect").

Our investigations show that the measurement of urinary total corticoids is a valuable parameter of adrenal function in mice which in some aspects may be more practicable than the estimation of plasma corticosteroids.

Acknowledgement—We gratefully acknowledge the skilled technical assistance of Miss I. Kloidt, Miss E. Major and Mrs. B. Soliman.

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