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Kinetics of Radiolabeled Adrenocorticotropin Hormone in Infant and Weanling Rats

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Abstract

Unlike the adult animal, the developing rat has a diminished ability to activate and inhibit the hypothalamic pituitary adrenal axis. In general, a gradual ACTH and corticosterone response to stressors appear after postnatal day 10 and is well established to adult level by weaning age. Although at this age the peak ACTH level is comparable to that of the adult, ACTH levels remain elevated for a longer period of time. The purpose of this study was to investigate the possibility that ACTH metabolism can, in part, explain this prolonged ACTH elevation after a challenge. The plasma half life of disappearance ($t_{1/2}$), the apparent volume of distribution and metabolic clearance rate (MCR) were determined after injection of a tracer dose of 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ in rats at 14 and 25 days of age. An adult animal group (65 days old) was used for comparison. The $t_{1/2}$ for ACTH decreases with age (14 day old $= 7.47 \pm 0.9$ min; 25 day old $=$ 6.48 ± 0.4 min; adult $= 4.46 \pm 0.2$ min) while the volume of distribution remains constant. The MCR is also decreased in the young animals (14 day old $= 1.5 \pm 0.19$ min; 25 day old $= 1.6 \pm 0.18$ min; adult $= 3.0 \pm 0.56$ min). For the first time, it is established that the young animals require longer to clear ACTH from an equivalent volume of blood when compared to the adult. Thus, the kinetic properties of ACTH are different in the developing animal and this partly explains the prolonged ACTH elevation observed after stress challenges.

The hypothalamic-pituitary-adrenal axis (HPA) in the developing rat has a limited response to acute challenges (1). After day 3 of life and until approximately post-natal day 14, rat pups fail to reliably increase plasma corticosterone levels to a variety of acute stressors, such as ether, handling and surgery. Based on this, the first two weeks of life are known as the Stress Hyporesponsive Period (SHRP). One key feature of this period is that the corticosterone levels rise with a specific time course which vary with age and with the specific stressor (2, 3). In general, a corticosterone (B) response to stressors is delayed and exhibits limited magnitude until weaning age (21–25 days old) when the adrenal response is well established to an adult level (1). This period of decreased corticosterone secretion is believed to be due in part to a decrease in adrenal sensitivity to circulating ACTH, although the mechanisms involved are not clearly known. Studies performed in neonatal animals have demonstrated that adrenal ACTH receptors are present on the adrenal cortex and these are coupled to adenyl cyclase (4). However, other investigators have shown that the decreased corticosterone secretion in early life may be dictated by a limited expression of cytochrome P-450 enzyme in the neonatal adrenal cortex (5), thus limiting steroidogenesis.

Much like the corticosterone response to stress, the magnitude of the ACTH response is also blunted during early life and increases steadily with age becoming equivalent to the adult response in the 25 day old animal (3, 6). Increases in the magnitude of the ACTH response parallels an increase in the hypothalamic corticotropin releasing hormone (CRH), vasopressin and pituitary ACTH reserve (7). The ACTH response to stress in the young animal is rapid, as in the adult, which has a peak ACTH response 5 min after the onset of stress. However, in the developing animal, unlike the adult, ACTH remains significantly elevated for a longer period of time after the initial challenge. This is clearly observed in the 10, 18 and 25 day old animals when exposed to ether vapor or cold temperature (3, 6, 8). Maternally deprived infant rats, which are made stress responsive during the SHRP, also show a sustained increase in stress induced ACTH levels after a saline injection which is unrelated to cold exposure (9). Several factors appear to explain this phenomenon. These include an immature adrenal which leads to a delayed and slow corticosterone rise that is not conducive to an appropriate fast feedback signal and a deficient regulation of the rate sensitive fast feedback mechanism at the pituitary and brain level (6). Another possibility is that the sustained ACTH increases observed after a stressor in both the maternally deprived infant rats and the weanling animals may reflect, in part, a reduced ability to remove this peptide from circulation. Since there is no information regarding ACTH clearance in developing rats, the present study was designed to determine the kinetics of ACTH in 14 and 25 days old animals and compare these to ACTH kinetics in the adult.

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Results

Experiment 1: Best fit curves of ACTH disappearance

The disappearance ACTH follows a logarithmical progression during the first 10 min after injection of 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$. As shown in Fig. 2, this pattern is observed in all ages studied, i.e. 14, 25 and adult animals. As shown by others, in the adult animal, ACTH disappears in two time phases (17–20). The first phase has a rapid fall, whereas the second has a more prolonged decay time. These time phases represent different pools of tissue distribution and also two different pools of hormones: bioactive and degradative products (17, 21–24). The first phase of the curve represents an inner pool, which reaches rapid equilibrium with the hormone in plasma. The second phase represents the hormonal pool which lacks biological activity (18, 23, 24) and that has a relatively slower equilibrium with the kidneys and liver (23–25). Because of these known components of the disappearance curve (17–20), pharmacokinetic calculations are performed, with the RSTRIP software program, based on the trapezoidal integration of the best fitted curve and the change in ACTH amount per unit time of the rapid component of the curve (for $t_{1/2}$ determination).

Pharmacokinetic parameters: half life, volume of distribution and clearance rate

The level of ACTH reached in plasma at time zero averaged 76.3 ± 5.3 fmoles/ml (mean of age 14, 25 and adult $\pm$ SE). Although the dose of radioactive ACTH was calculated based on BW the percent of the injected dose reaching plasma varied with age (14 day old $= 10.3 \pm 0.9$; 25 day old $= 6.9 \pm 0.7$; adult $= 5.3 \pm 0.3$; % mean $\pm$ SE). Tissue absorption and plasma distribution at different ages may account for these differences. In fact, these differences are evident in Table 1 which shows that 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ metabolism and its plasma distribution varies with age. It takes one and a half times longer for 50% of the 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ to decline in the young animals when compared to the adult (Table 1—$t_{1/2}$ values). This is, in part, reflecting the apparent volume of distribution (V_d) which in the immature animals, is larger despite its smaller body size (Table 1—V_d; see reference 17). Similarly, although calculated by a different method, the 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ metabolic clearance in the 14 and 25 day old animals is half of that which is seen in the adult (Table 1—MCR; $P \leqslant 0.05$). Thus, the pharmacokinetic parameters, especially $t_{1/2}$ and MCR, suggest that ACTH concentrations are eliminated slowly in the immature animal. Would the metabolism of ACTH alone explain the prolonged ACTH profile observed in the young animal after a pituitary challenge? To answer this question, plasma ACTH levels were measured in the 25 day old and adult animal after exposure to ether vapors. Because of ACTH levels were measured by RIA in distinct time points, the ACTH profile obtained were corrected using the half life values derived from the 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ kinetic study. This application is done with caution since it is possible that the clearance of ACTH is different in the anesthetized animal when compared to the free moving animal.

Experiment 2: Pituitary and adrenal response to ether vapors

In both ages, the peak ACTH level is of similar magnitude and it is observed 5 minutes after exposure to ether vapors (see Fig. 2, Panel A). Corticosterone levels were also measured in these

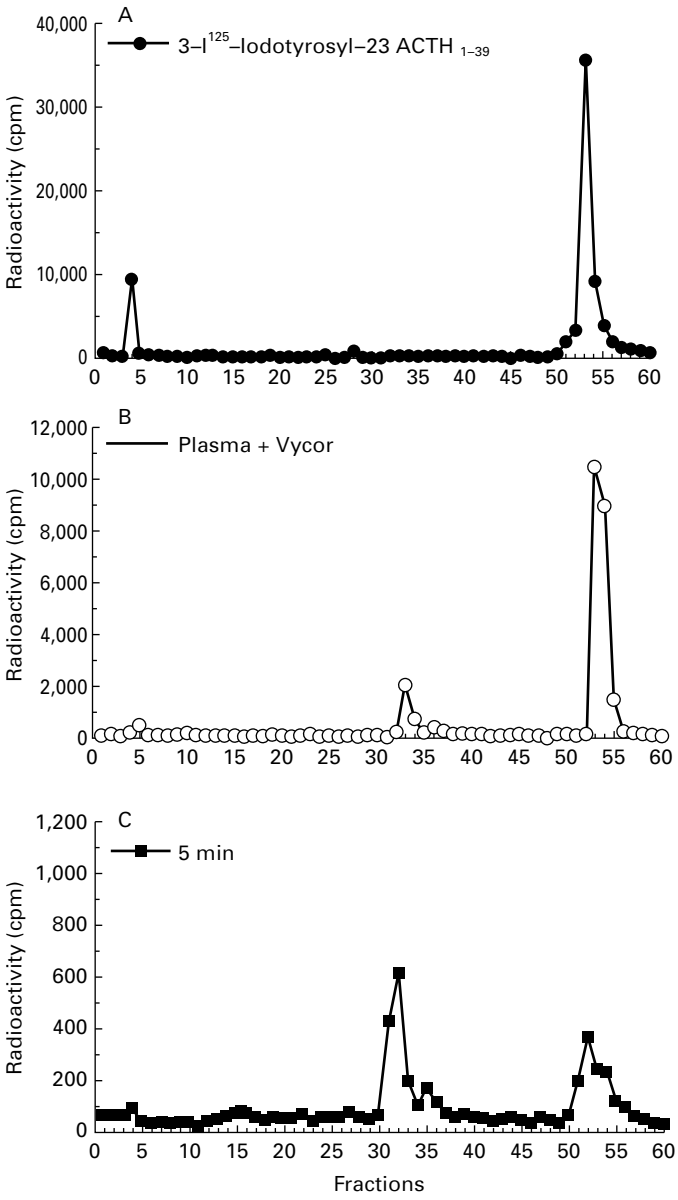

FIG. 1. High Pressure Liquid Chromatography profile of the radioactive products originating from the 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ which were attached to the glass silica used for the plasma extraction method. The peptides were extracted from the glass silica with 40% acetonitrile: 0.1% triflouroacetic acid and separated using acetonitrile and 0.1% triflouroacetic acid in a Beckman Model HPLC system. The samples processed were: (A) sample containing 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ prior to the administration and not extracted with the glass silica, (B) 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ and extracted with the glass silica (Vycor) and (C) a sample taken 5 min after injection of material to an adult animal also extracted with glass silica. Early (fractions 3–5) represents free iodine and a second peak in fractions 50–60 represents the intact molecule. Pure 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ mixed with plasma and subjected to the glass silica extraction eluted in samples 5, 32–35 and 52–57 (Panel B). Five min after injection of radioactive material to an adult animal, a glass extracted plasma sample shows a profile of 3 peaks between fractions: 31 to 34, 34 to 37 and 50 to 56 (Panel C), suggesting processing of molecule *in vivo*. Thus, the glass extraction procedure minimizes free iodine in the sample and removes large 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ fragments from plasma (11).

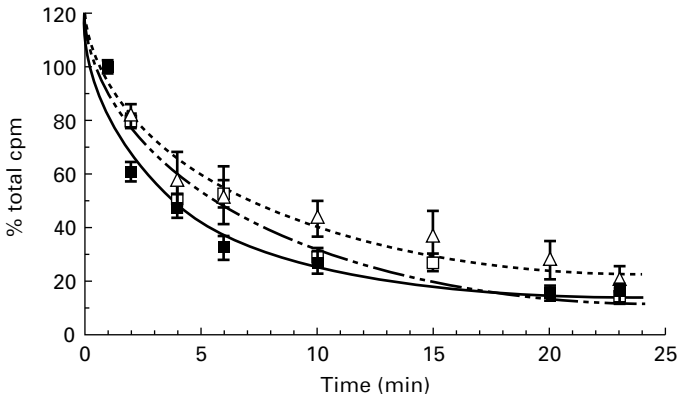

FIG. 2. Disappearance of labeled hormone with time from plasma of individual rats age, 25 days, 14 days and adults, injected with 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$. Values are mean ± SE. The following number of animals were used in each age group: 25 days = 6, 14 days = 6 and adults = 13. □ 25 day old; △ 14 day old; ■ Adult.

animals (see Fig. 2A, insert) and they are consistent with previous reports (2, 3, 6). In the 25 day old animal the CORT release is delayed when compared to the adult (peak level, adult = 15 min *vs* 25 day old = 30 min). In the 25 day old rat, the ACTH level remains significantly elevated for a longer period of time (P = 0.002 by two factor repeated measure ANOVA). In fact, the ACTH level is elevated 45 min longer than in the adult animal. In experiment 1, we estimated that the adult ACTH half life is 4.5 min. The adult ACTH profile presented in Figure 2, Panel A, is consistent with this value. If 50% of the ACTH level present in the 25 day old animal were to decline as in the adult (50% every 4.5 min), the ACTH response to ether vapor becomes identical to that of the adult animal (see Fig. 2, Panel B; $t_{1/2}$ = 4.5 min). It is also evident from Figure 2 that prolonged ACTH metabolism does not entirely explain the elevated ACTH response in the 25 day old animal. As can be seen, correction of the 25 day old plasma response profile using the half life value for this age shows a slightly delayed response, but the delay is minor, compared to what is in fact observed in 'real life'. Thus, in the 25 day old animal ACTH remains elevated beyond the time predicted by considering clearance alone (see Fig. 2, Panel B; $t_{1/2} = 6.5$).

Discussion

The turnover characteristics of ACTH have been studied in several adult organisms including: rodents, sheep, dogs and humans (14, 17, 18, 20, 23–31). To our knowledge, the metabolism of ACTH has not been studied in developing organisms. The present study was designed to address whether sustained ACTH elevations seen in the developing rat after a stressor may be due to a slow clearance of the ACTH peptide from blood. The results of this study reveal that the ACTH disappearance time and metabolic clearance is significantly prolonged in the developing animal when compared to the adult.

Investigations of hormone clearance can be performed after a rapid single injection or a continuous infusion of radioactively labeled or unlabeled hormone. Both, rapid single injection (18–20, 25) and continuous infusion with unlabeled or labeled hormone (14, 31) have been used to study the rate of disappearance and removal of ACTH molecules from circulation in the adult rat. These methods achieve either a transient or a steady state elevation of the hormone in plasma. Regardless of the method of administration, constancy and linearity of metabolic clearance is achieved when high ACTH plasma concentrations are achieved. This is true in adult rats with stress levels of ACTH (18, 19) and animals injected or infused with large doses of ACTH which achieve plasma levels greater than 0.258 ng/ml (56.8 fmol/ml) (14, 31). Under these conditions, tissue receptors are saturated and corticosterone levels are maximal and steady, which lead to ACTH half life, volume of distribution and metabolic clearance determinations which are consistent across studies (21, 23). With this in mind, the animals in our study were injected with an equivalent ACTH dose which resulted in comparable plasma ACTH levels (76.3 ± 5.3 fmol/ml). Thus, the plasma level achieved is in the order of the ACTH concentrations that is associated with constancy of metabolic parameters.

Another consideration is the choice of ACTH molecule administered to the animals and the method of detection of its decay in plasma. The ideal molecule to study is a physiologically meaningful molecule which would be endogenously synthesized and bioactive. Historically, the fate of ACTH molecules has been studied by several groups using synthetic $ACTH_{1-24}$ or $ACTH_{1-39}$ as radiolabled or non-radiolabled forms. The $ACTH_{1-39}$ molecule was selected in the study presented here due to its stable properties in plasma and the fact that its metabolism would lead to the formation of the major ACTH form identified in the rodent: $ACTH_{1-31}$ phosphate (32, 33). Previous reports have shown that the radioactivity and immunoreactivity of $ACTH_{1-39}$ parallel each other, while the bioactivity decreases at much rapid rate (18) corresponding roughly to the first phase of the immune and radioactive curves [$t_{1/2}$ immuno method = 2.0–6.2 ml/min (17, 18, 23); $t_{1/2}$ bioactive method = 2.3–5.5 ml/min (18, 20, 23, 26)]. This relationship has lead to the opinion that, in general, the difference

TABLE .1 Pharmacokinetic Parameters: Half Life ($t_{1/2}$), Apparent Volume of Distribution (V/d) and Metabolic Clearance Rate (MCR) of 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ in Plasma of Animals at Different Ages.

Age	BW (g)	Half life ($t_{1/2}$) min	Apparent volume of distribution (V_d) ml·50 g BW	Apparent volume of distribution (V_d) ml	Metabolic clearance rate (MCR) ml·min
14 days (6)	36.2 ± 0.97	7.47 ± 0.9*	19.7 ± 0.5*†	14.2 ± 0.3	1.5 ± 0.19*
25 days (6)	50.7 ± 0.49	6.48 ± 0.4*	14.1 ± 0.1*	14.3 ± 0.002	1.6 ± 0.18*
adult (13)	339.1 ± 6.6	4.46 ± 0.2	3.1 ± 0.9	21.3 ± 5.8	3.0 ± 0.56

Mean ± SE. The number in parenthesis indicate the number of animals in each group. *significatnly different from adult, $P < 0.05$. †significantly different from 25 day old, $P < 0.05$.

between bioactive and immune methods is due to the fact that antibodies used in the immunoassays are likely to recognize the C-terminus of the molecule, while bioassays are sensitive to the N-terminus. Transformation to bioinactive forms may also entail relatively minor structural changes with no loss of immune detection. Thus, both biologically active and non-biologically active ACTH fragments are quantified with the radioactivity decay or the radioimmunoassay methods. However, these methods can give accurate information on the disappearance of the relevant ACTH molecules from plasma, specially if the first phase of the curve of disappearance is analyzed. In our study the radioactive method was prefered for analysis due to the size of the animals which limited the total amount of blood that could be retrieved without significantly depleting the total blood volume. In addition, the radioactive measurement of 3-I^{125}-Iodotyrosyl^{23} $ACTH_{1-39}$ avoids the measurement of endogenous ACTH secreted during the surgical procedure.

In our study we found good agreement among kinetic parameters obtained from our adult animals and those reported in the literature. Indeed, we find an ACTH half life of 4.5 min and this is consistent with previous studies which report ACTH half life ranging between 4.1 to 6.2 min (17, 18, 23). The calculated metabolic clearance rate is also comparable with that which has been reported: 3.6 ± 0.6 ml/min/100 g BW for low plasma ACTH concentrations down to 1.0 ml/min/100 g BW for higher ones (14, 19). Since high plasma ACTH concentrations were maintained, our adult animals clear 0.88 ml of plasma ACTH in one minute per 100 g of body weight. Therefore, there is very good agreement between what is reported in the literature and the kinetic parameters obtained from our adult animals. This leads us to propose that these parameters can be accurately calculated in the younger animals using the radioactive decay method. Our data are consistent in showing a longer time of ACTH disappearance in the younger animals when compared to the adults. The half life is approximately one and a half longer than in the adult. This is consistent with a slower clearance of the hormone since the young animal takes twice the time the adult to completely and irreversibly remove ACTH from three ml of blood (14 and 25 day old MCR $\sim$ 1.6 ml per min *vs* adult MCR = 3.0 ml per min). A possible explanation for a slower ACTH clearance in the young animals is that the method of radioactive decay may have the intrinsic problem of the ACTH molecule losing its iodinated marker prior to its processing. If this were to be occurring at a slower rate in the young when compared to the adult it would explain the slower clearance observed at a young age. However, comparison of the radioactive decay method with immunoassay methods in the adult animal suggest that if the ACTH molecule is losing its iodinated marker, it is a slow process which does not alter significantly the results obtained from either method (18, 20, 23, 26). Since we are obtaining a slower clearance in the young animals when compared to the adult, this suggests that the loss of the iodinated label in the young would be much slower or negligible. Therefore, this phenomena does not explain our findings.

We would predict the clearance of ACTH to be slower in the developing animal. This is based on the fact that circulating ACTH is removed and catabolized by various tissues whose functions are in a state of flux in the developing animal (34–38). This is particularly true of the renal and hepatic systems which appear to be involved in the clearance process of many hormones (39–40). In the adult animal, 1 min after injection ACTH is distributed to plasma, muscle, skin, gut, kidney and liver (23, 25). Muscle and skin accounts for 50% of the ACTH distribution. In less than 5 min post injection peak levels are observed in plasma and adrenal. However, peak levels are attained slower in kidneys and liver but represent a significant amount (27% of total in kidneys and 12% in liver) (23, 25, 34). This is probably due to the fact that, as with other peptide hormones, these organs are involved in the uptake and metabolism of ACTH (18). It has been documented that plasma clearance of ACTH is lower in rats with ligated kidneys or after partial hepatectomy (40–42). Of these 2 systems, renal ACTH processing is the best studied. Specifically, experiments performed in adult animals reveal that radiolabled ACTH is absorbed into proximal tubules cells of the kidney by endocytosis to produce vesicles that were later found in lysosomes where peptidases degrade it into amino acids or peptic fragments (22, 39). This is an irreversible clearance process, the rate of which is determined by glomerular filtration. During the first weeks of life, the glomerular filtration rate (GFR) is decreased in the developing animal as a function of a decreased blood flow to these organs (37, 38). Thus, we reason that a decrease in GFR may explain the fact that the younger animals clear half as much blood volume of ACTH per unit time as does the adult animal. Alternatively, the interaction of the ACTH iodinated molecules or its metabolites with membrane receptors in the young may not be condusive to proper uptake and metabolism of ACTH as observed in the mature animal.

Is this reduced ability to remove ACTH peptide responsible for the sustained ACTH levels observed in the developing animal exposed to stressors? To clarify this, we applied the kinetic data to an ACTH profile obtained after ether vapor in 25 day old and adult animals. The most conservative kinetic measure, half life of ACTH disappearance, was the kinetic parameter used in view of the fact that the time variable could easily be applied to the peak ACTH response obtained after a stressor. What we observe is that based on the $t_{1/2}$ value obtained from the 25 day old animal, the ACTH profile should be very similar to the adult animal. The 25 day old animal should decrease its circulating plasma level to a baseline level within 15 to 20 min after exposure to ether vapor. Instead, ACTH secretion continues for much longer. Consistent with previous reports this prolonged ACTH secretion is associated with a delayed adrenal corticosterone response (2, 3). In effect, in the 25 day old animal the CORT release is delayed by 15 min when compared with the adult (peak level = 15 min in the adult *vs* 30 min in the 25 day old). A previous study has shown that even if the delay is corrected in the young animal by administering glucocorticoids that peak at 15 min after ether exposure, the ACTH secretion is blunted but continues to be sustained for 30 min (6). Thus, in the 25 day old animal, the delay to return to resting ACTH levels is not completely accounted by the time delay observed for the CORT release from the adrenal. This is unlike the adult in which glucocorticoid administration will inhibit ACTH secretion within 15 min after the stressor (6). In the present study, we find that correction of the 25 day old ACTH plasma response profile using the half life value for this age does not result in a sustained ACTH response, but rather approximates the ACTH profile substantially to that of the adult. This suggests that in the 25 day old animal, ACTH continues to be secreted for a prolonged period of time after ether exposure when compared to the adult and although a slow ACTH clearance may

contribute to this pattern, the greater contributor is a deficient rate sensitive fast feedback mechanism.

In conclusion, the development of a competent and fully functional hypothalamic-pituitary-adrenal axis surpasses the initial postnatal period in the rat. The animal acquires the ability to activate a stress response before the weaning period, but ACTH levels remain elevated for a longer period of time after certain stressors. A diminished clearance of circulating ACTH partly explains the elevated ACTH levels observed in the immature animal following a stress challenge. An immature adrenal response coupled with a deficient regulation of the rate sensitive fast feedback at the pituitary and/or brain level are greater contributing factors to this phenomena.

Material and methods

Animals

Sprague-Dawley female rats (Charles Rivers, Wilmington, MA, USA) were mated in our animal unit. Animals are maintained in accordance with the NIH guidelines for the Care and Use of Laboratory Animals. Pregnant rats had free access to food and water and were kept on a 14 h light, 10 h dark cycle. Vaginal plugs marked the first day of pregnancy. On the day of delivery, which was considered day one of life, the pups were randomized across litters, reduced to 8 pups per litter (1 : 1 sex ratio) and were left undisturbed until the day of experimentation. A total of 26 litters were used for these experiments to ensure genetic diversity. Those male pups which were going to be used when 25 day old, were removed from their mother on day 21 of life and grouped six animals per home cage. Adult males (65 days old) were also born in our breeding facility. They were housed 3 animals per home cage with free access to food and water. All experiments were carried out in the morning, between 08.00 and 11.00.

Experiment 1. 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ injections, blood sampling and assay

Adult (n = 13), 14 (n = 6) and 25 day old (n = 6) male Sprague Dawley rats were injected with Nembutal (50 mg/kg), which maintained the animals anesthesized throughout the bleeding session. Laparotomy was performed in the animals and a polyethylene cannulae was implanted in the abdominal inferior vena cava for blood withdrawal. Cannulation was performed between 2 to 3 min in all animals followed by heparinization with 20 units of heparin per 100 g body weight (BW). Synthetic ACTH in the form of 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ (Amersham, CA 2000 Ci/mmole) was resuspended in 1 ml of 0.9% saline/1N acetic acid. This peptide was preferred over the αACTH$_{1-24}$ because of its stable qualities in solution. Animals received an equivalent dose of 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ calculated to give 0.4 μCi per 100 g BW, which reached a plasma ACTH level of 76.3 ± 5.3 ng/ml (mean ± SE, see below). The dose was administered as a venipuncture to the inferior phrenic vein-inferior vena cava juncture at the level of the diaphragm. The time between cannulation and injection of trace was consistently within 1 min for all animals. Blood samples were obtained via the abdominal inferior vena cava heparinized catheter at 1, 2, 4, 6, 10, 20, and 30 min after the injection. The animals were sacrificed after obtaining the last blood sample. The procedure was performed between 09.00 and 10.30 in the morning over several days. For each session two adult and two young male animals (14 and 25 day old) were cannulated and body temperature was maintained at nest temperature with a heating pad (33 °C). Based on an estimate of total blood volume (BV-D14 = 2.69 ± 0.01; D25 = 3.77 ± 0.1 and adult = 25.25 ± 0.5; ml ± SE) and plasma volume (PV-D14 = 1.66 ± 0.1; D25 = 2.31 ± 0.04 and adult = 15.60 ± 0.7; ml ± SE, see ref. 10), an equal percentage of blood volume was retrieved from each animal. Thus, blood sampling consisted of 0.5 ml per time point from the adult and 0.1 and 0.2 ml from the 14 and 25 day old animal respectively. The samples were collected in chilled EDTA containing tubes and later centrifuged at 4 °C to separate plasma. Fifty microliters of plasma were diluted with 500 μl of distilled water. The I^{125} ACTH$_{1-39}$ was extracted from plasma vortexing the mixture with 50 mg leached glass silica (Corning Glass Co. #7930) as described by Rees *et al.* (11). The supernatant fluid was decanted, the glass silica was washed with 1 ml of distilled water and counted directly in the gamma radioactivity counter. In addition, the counts were corrected for the 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ recovery from the glass silica. Recovery was found to be 89% ± 3.3 with this method (n = 6).

High pressure liquid chromatography (HPLC)

The radioactive products attached to the glass silica were analyzed to ensure that only fragments originating from the 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ were being counted by our crude extraction method (11). The peptides were extracted from the glass silica with 40% acetonitrile: 0.1% triflouroacetic acid. The HPLC system was composed of the following: 2 Beckman Model 110 pumps with a 421 system controller, a Rheyodyne injector with a 1 ml loop, a 4 mm × 26 mm Vydac Protein/Peptide 0–18 column, and a Pharmacia Frac 100 fraction collector. The solvents employed were acetonitrile (Solvent B) and 0.1% triflouroacetic acid (Solvent A) disolved in water. Solvent B was increased from 10% to 70% over a 120 min gradient. The sample injection volume was 1.0 ml. One mililiter elution samples were collected at one minute intervals using a Pharmacia Frac 100 fraction collector and counted on a Gamma Trac 1290 gamma counter. Three samples were processed: A) a sample containing 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ prior to the administration and not extracted with the glass silica; B) a sample of 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ mixed with fresh plasma which was extracted with the glass silica and C) a sample obtained 5 min after injection to an adult animal. The extraction column separated two peaks from the 'pure' 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ sample which eluted early (fractions 3–5) and represents free iodine and a second peak in fractions 50–60 represented the intact molecule (Panel A, Fig. 1). Pure 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ mixed with plasma and subjected to the glass silica extraction eluted in samples 5, 32–35 and 52–57 (Panel B, Fig. 1). Five min after injection of radioactive material to an adult animal, a glass extracted plasma sample shows a profile of 3 peaks between fractions: 31 to 34, 34 to 37 and 50 to 56 (Panel C, Fig. 1). Thus, contact with plasma appears to generates a radiolabeled species that is processed *in vivo* to several forms. More importantly, as previously shown by Ress and co-workers, the glass extraction procedure minimizes free iodine in the sample and removes large 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ fragments from plasma (11).

Calculation of pharmacokinetic parameters

The 3-I^{125}-Iodotyrosyl^{23} ACTH$_{1-39}$ disappearance curve was best fit to equations derived from compartmental modeling and kinetic analysis using RSTRIP Program (Micromath Scientific Software™, Salt Lake City, Utah, USA). RSTRIP employs a least squares minimization procedure based on a modification of Powell's algorithm (12, 13). Using this analytical program, the best exponential fit of the data set generated from each individual animal is determined, a calculated curve is displayed and the half-life ($t_{1/2}$) of the administered substance is estimated. The program makes a statistical moment analysis using trapezoidal integration which provides the values for the areas under the zeroth curve (AUC) and area under the first moment concentration curve (AUMC). The zeroth curve is plotted from the mathematical derivation to the curve to time 0. The moment concentration curve's first point corresponds to the first time of blood collection (1 min). The AUC and AUMC calculation take into account coefficients and rate constants as follows: $AUC = \sum A_i/k_i$ and $AUMC = \sum A_i/k_i^2$. Once calculated, the AUC and AUMC values can be used to directly calculate the metabolic clearance (MCR) and apparent volume of distribution (Vd) without presumption of a specific underlying compartmental model (12). Clearance is calculated using the following formula: $MCR = D_{iv}/AUC$; where D_{iv} is the fraction of the administered intravenous dose of radiolabled ACTH reaching the site of measurement (extrapolated from the curve at time zero) multiplied by the administered dose. Based on the zeroth curve the administered ACTH reached an averaged level of 76.3 ± 5.3 ng/ml (mean ± SE). Levels greater than 10 ng/ml are associated with maximal CORT response and with constancy of metabolic parameters (14). The apparent volume of distribution (V_d) is computed by RSTRIP with the formula $V_d = D_{iv}\ AUMC/(AUC)^2$.

Experiment 2. Pituitary and adrenal response to ether vapors

Twenty five day old male animals were removed from their home cage in groups of six (as they were housed in groups of 6) and male adults were removed in groups of three (as they were housed in groups of three). To ensure genetic diversity for each time point, the animals were obtained

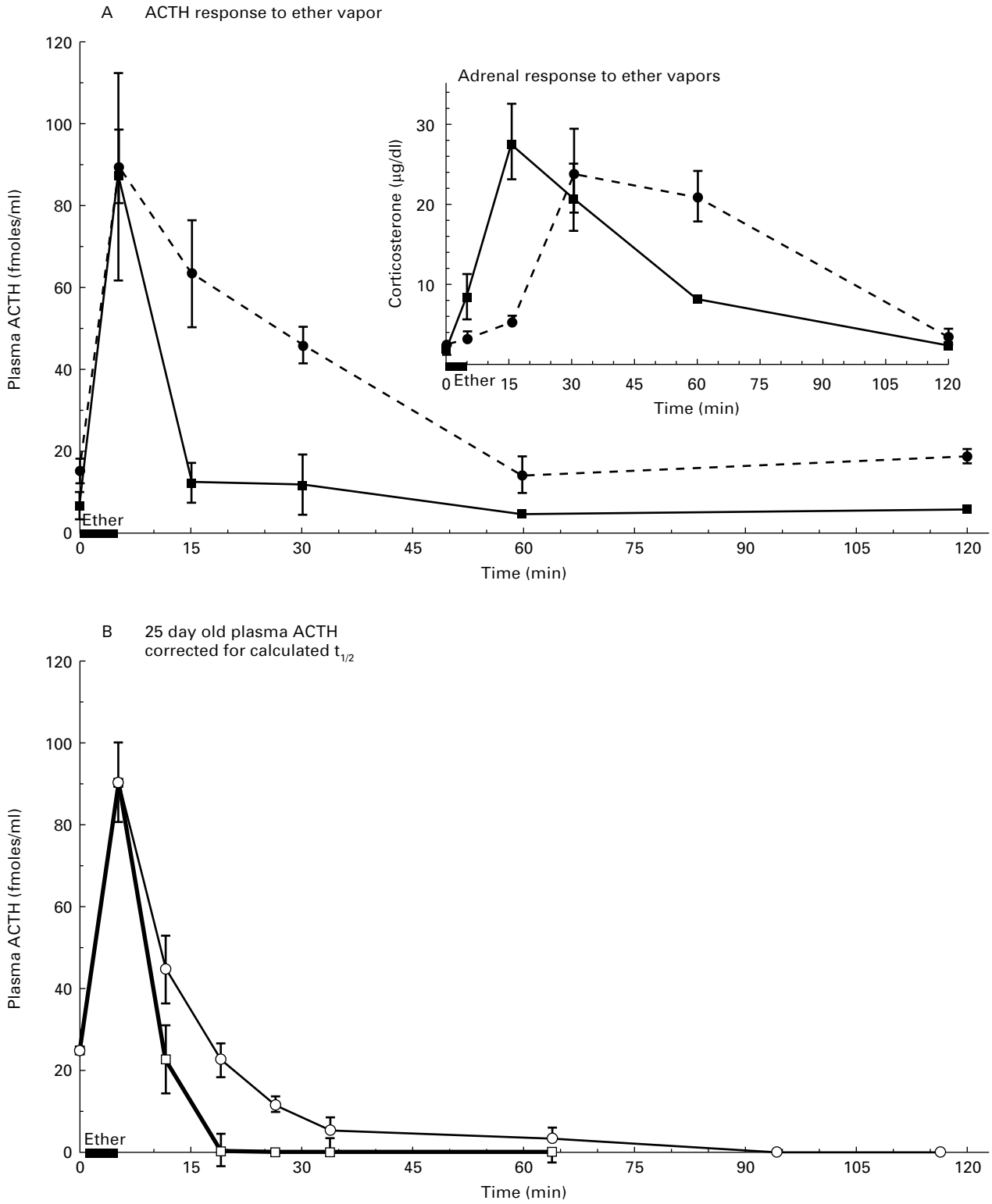

FIG. 3. Time course of the plasma ACTH and corticosterone secretion after 5 min exposure to ether vapor. The 25 day old response is compared to the adult response in Panel A. Corticosterone response can be seen in the insert. Panel B shows the ACTH plasma profile of the 25 day old when the ACTH half life corresponding to the adult or when the ACTH half life corresponding to the 25 day old is applied to the original pattern of secretion. Panel B is derived from the data presented in Panel A and shows that a prolonged ACTH metabolism does not entirely explain the elevated ACTH response in the 25 day old animal. A ■ Adult; ● 25 day old; B □ $t_{1/2}$=4.5 min (adult $t_{1/2}$); ○ $t_{1/2}$=6.5 min (25 day old=$t_{1/2}$).

from 18 different litters. They were transferred to an adjacent experimental room where they were immediately placed in a glass jar containing gauzes saturated with ether below a wire mesh platform. The animals were maintained in the closed container for 5 min, sufficient time to anesthetize all of the animals present in the jar. They were then returned to their home cage. All animals were sacrificed by decapitation at 5, 15, 30, 60 or 120 min after being placed in the container. Trunk blood was collected in EDTA containing tubes for plasma ACTH determination. The sacrifice were done between 09.00 and 10.30 in the morning. Six animals of each age were sampled in each time point throughout this timeframe. In a similar fashion, from 09.00 to 10.30, eight unhandled animals were also sacrificed to estimate basal ACTH level (time 0).

Plasma Assay-Plasma ACTH Assay. Plasma ACTH is measured by RIA which has been described in previous reports (15, 16). The antibody used cross-reacts less than 1% with alpha MSH, desacetyl MSH and has no cross-reactivity with $ACTH_{17–39}$ (CLIP). The detection limit is 1 fmol per ml (intra coefficient of variation $\pm$2%, inter assay coefficient of variation $\pm$3%).

Plasma Corticosterone Assay. Plasma corticosterone is measured by RIA, it utilizes a specific rabbit antibody (B3), a generous gift of Fernando Estivaríz (La Plata, Argentina) at a final concentration of 1:4,000 (6). The antibody cross-reacts 2.2% with cortisol and less than 1% with other endogenous steroids. The radioligand is ^{3}H corticosterone (Amersham 50 Ci/mmole) and 1% charcoal/0.1% dextran mixture is used for the separation of the bound and unbound phase. The detection limit is 1 pg per ml (intra coeficient of variation $\pm$3%, inter assay coeficient of variation $\pm$2%).

Statistical Analysis. Analysis of variance was used to determine the significance of differences between means of the pharmacokinetic parameters. In the case of ACTH and corticosterone response to ether vapor, two factor ANOVA was used for the analysis of time interactions. Post-hoc comparisons of treatment groups were made with Fisher PLSD. Values are expressed as mean $\pm$ SE.

Abbreviations: adrenocorticotropin hormone, ACTH; hypothalamic-pituitary-adrenal axis, HPA; stress hyporesponsive period, SHRP; metabolic clearance, MCR; apparent volume of distribution, Vd; half-life, $t_{1/2}$; areas under the zeroth curve, AUC; first moment concentration curve, AUMC.

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