Developmental Expression of the Peripheral-Type Benzodiazepine Receptor and the Advent of Steroidogenesis in Rat Adrenal Glands*

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ABSTRACT

Although the precise mechanism whereby cholesterol is transported across the outer mitochondrial membrane is uncertain, a multimeric receptor complex termed the peripheral-type benzodiazepine receptor (PBR) appears essential for this process. We therefore predicted that adrenal cells at different developmental stages would express PBR coincidentally with the advent of steroidogenesis. Adrenals of neonatal rats demonstrate greatly reduced sensitivity to ACTH that gradually increases after the first 2 weeks of life. Thus, neonates have lower circulating corticosterone levels following exposure to stress. We examined mitochondrial PBR ligand binding activity, immunoreactive (ir) PBR content, and adrenal sensitivity to ACTH in vivo and in vitro. Ontogeny of both mitochondrial PBR ligand binding capacity and irPBR directly paralleled that of ACTH-inducible steroidogenesis in isolated rat adrenal cells and in rats injected with ACTH. In addition, neonatal PBR had approximately 2-fold higher affinity for PK11195, a synthetic ligand that binds with high affinity to PBR. No correlation was observed during neonatal life between ir-steroidogenic acute regulatory (StAR) protein content and steroidogenesis. These results are consistent with the hypothesis that PBR is an absolute prerequisite for adrenocortical steroidogenesis, and suggest that the stress hyporesponsive period of neonatal rats may result from decreased PBR expression. In addition, the higher affinity of neonatal PBR and the relatively high basal expression of StAR protein in neonatal adrenals may partly explain the high constitutive steroidogenesis characteristic of neonatal rat adrenal cells. (Endocrinology 140: 859–864, 1999)

The rate-limiting step in steroidogenesis is transport of free cholesterol from intracellular stores to the inner mitochondrial membrane, where the first steroid biosynthetic enzyme is located (cytochrome P450scc). The process whereby cholesterol is generated and targeted to the mitochondria begins with the generation of cAMP following binding of ACTH to its membrane receptor. The precise mechanism whereby cholesterol reaches the outer mitochondrial membrane, and is then transported to the inner membrane at contact points within the mitochondria, remains uncertain.

In recent years, it has become clear that at least two intracellular molecules assist in mediating the cholesterol transport process. The first of these, known as the steroidogenic acute regulatory protein (StAR), is a 30-kDa protein whose synthesis is stimulated by ACTH, and which associates with mitochondria (1). StAR is believed to act by promoting the targeted movement of cholesterol to relevant sites on the outer mitochondrial membrane (1), and is found in all steroidogenic cells except for those in placenta and brain (1, 2). The second molecule is actually a complex of molecules collectively known as the peripheral-type benzodiazepine receptor, or PBR. This membrane-spanning receptor, found on the outer mitochondrial membrane of all steroidogenic cells (3), gets its name from its ability to bind natural and synthetic ligands of the benzodiazepine family. Targeted disruption of the PBR gene in mouse Leydig tumor cells essentially eliminates steroidogenic capability (4), which is restored with replacement of an active PBR complex (4). Unlike StAR, PBR is constitutively expressed in steroidogenic cells (3). Activation of PBR with endogenous or synthetic ligands facilitates steroidogenesis, however, suggesting that the molecule is under regulatory control (3, 5–7).

If both or either of these regulatory molecules are essential for steroidogenesis, we hypothesized that the level of expression of StAR and/or PBR would correlate with changes in steroidogenic capability of a particular steroidogenic gland during an animal’s life history. During neonatal life in rats, for example, the adrenal cortex undergoes a period of about 2 weeks during which constitutive steroidogenesis is relatively high (compared with cells from adult animals, in which such activity is absent or negligible) (8, 9), but ACTH-responsiveness is very low (8–11). Although changes in ACTH-responsiveness may be partly related to developmental changes in microsomal steroidogenic enzymes (12), the mechanisms of both the constitutive process and the lack of ACTH-responsiveness remains largely unknown. We predicted that StAR and/or PBR expression would be low during the neonatal period, and that if true, this could provide an explanation for the reduced adrenocortical steroid output during this period.

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Materials and Methods

Animals

Male Sprague Dawley rats were purchased from Holtzman (Madison, WI). For studies with neonates, timed pregnant females arrived at our animal care facility at 18 days of gestation. Pups were kept with their mothers until immediately before use. Both male and female pups were included in all experiments. All experiments were approved by the Boston University Institute Animal Care and Use Committee.

ACTH injections

On the day before an experiment, animals were transferred to a controlled quiet room and given 24 h to acclimate to their surroundings. All experiments began at approximately 0900–1100 h (lights on 0700–1900 h). One group of untreated adult male (−200–250 g) or neonatal (both sexes) rats was killed by decapitation at the start of the experiment to collect baseline data for hormone and PBR measurements. Additional groups of rats were injected ip with 250 μl (adults) or 100 μl (neonates) physiological saline or porcine ACTH(1–39) (Sigma Chemical Co.) at a dose of 10 μg/kg, except for postnatal day (pd) 10 pups, which received 20 μg/kg, and returned to their home cages. These doses were chosen on the basis of previous experiments (10), and preliminary trials that indicated their effectiveness in elevating circulating ACTH to high physiological levels for approximately 2 h. It is unknown at present why d10 pups required a higher dose of ACTH to achieve the same circulating levels reached by the lower dose at all other ages tested, but it is possible that this is related to age-dependent changes in clearance of ACTH.

Groups of approximately 10 (neonates) or 6 (adults) animals were killed in random order (saline vs. ACTH-injected) at each of several selected times after injection. The blood was collected into EDTA (final concentration ~1–2 mm) and centrifuged; plasma was frozen in aliquots for corticosterone and ACTH RIAs. Adrenal glands were immediately dissected from the carcasses, decapsulated to remove the outer glomerulosa/capsule layer, and homogenized on ice for 45 sec in ice-cold Tris (50 mM)/sucrose (0.25 M) buffer, pH 7.4, with a TekMar tissue grinder. Mitochondria were prepared by differential centrifugation as previously described (12), and stored frozen for future immunoblot and binding analyses.

Cell secretion experiments

Adult male (−450 g) or neonatal (both sexes; pd 9–11) rats were killed by decapitation, and the adrenals immediately dissected. The right adrenal glands from each animal were pooled and used for in vitro analysis of ACTH sensitivity (see Fig. 1); the left adrenal glands were pooled, homogenized as above, and mitochondria prepared for PBR analysis (see Table 1). For cell secretion studies, the right adrenals from 12 (adult) or 20–60 (neonates) rats were pooled, minced, and subjected to enzymatic dispersion as previously described (9). This process was repeated in three different experiments. Approximately 100,000–150,000 viable cells with large vacuolar structures consistent with the appearance of lipid droplets were incubated in 1 ml of Medium 199 (with 15 mM BSA) (13) for 2 h in a humidified, oxygenated environment. Porcine ACTH(1–39) or vehicle was then added directly to the tubes in a volume of 10 μl, and the incubation was continued for an additional 2 h. The cell suspension was centrifuged and the supernate assayed for corticosterone by RIA. Each point is the mean and SEM of observations from three separate experiments, each performed in duplicate or triplicate. Results are normalized to the number of viable (dye-excluding), lipid droplet-containing cells in the suspension. The two curves were significantly different when assessed by two-factor ANOVA.

Immunoblot analysis

Adrenal mitochondrial proteins from the unstimulated rats described above in the injection study, were fractionated by one dimensional SDS-PAGE on a 15% acrylamide gel. The proteins were then transferred onto 0.45 μm nitrocellulose membranes (Schleicher & Schuell, Keene, NH) at 0.9 A for 30 min using a Trans-Blot Cell (Idea, Corvalis, OR). Nonspecific adsorption of the antibodies was blocked by incubating the nitrocellulose in 5% milk. The blots were then treated for immunodetection of PBR, stripped, and reblotted for detection of StAR protein using anti-PBR and anti-StAR at 1:1000 dilution. Anti-peptide antiserum to amino acids 71–88 of PBR and to amino acids 89–107 of the 30 kDa StAR protein were prepared as previously described (14). Goat IgG-horseradish peroxidase was used as secondary antibody at 1:6000 fold diluted.

Additional aliquots of mitochondria (10 μg protein) prepared above were resuspended in PBS and used for Scatchard analysis of PBR binding activity. [N-methyl-3H]PK11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isooquinolinecarboxamide; specific activity 83.5 Ci/mmol; DuPont NEN) binding studies were performed at 4 C, in a final incubation volume of 0.3 ml, using the radioligand at a concentration range of 0.05–12.5 nm (10–14 concentrations/assay) and 1000-fold excess of unlabeled ligand (Research Biochemicals International Inc., Natick, MA), as previously described (5, 14–16). After 120 min incubation, assays were stopped by filtration through Brandel GF/C glass fiber filters and washed with 5 × 5 ml ice-cold PBS. Radioactivity trapped on the filters was determined by liquid scintillation spectrometry at 30% counting efficiency. The dissociation constant (Kd) and the number of binding sites (Bmax) were determined by Scatchard plot analysis of the data using the LIGAND program (17) (KELL, version 4.0, Biosoft, Inc.).
Corticosterone and ACTH were determined by RIA according to the manufacturer’s specifications (ICN), except that volumes of all reagents were reduced by 50%, and cell suspension supernates were diluted 1:1000 for adult cells and between 1:10 and 1:100 for neonatal cells before assay, as previously described (13). Statistical analysis was by one- or two-factor ANOVA with Bonferonni correction. Regression analysis and areas under the curve were performed using the Prism software program from GraphPad, Inc. Protein was determined by the dye-binding assay of Bradford (18) with γ-globulin as standard.

Results

Acutely isolated adrenocortical cells from neonatal (pd9–11) rats were significantly less sensitive to ACTH in vitro than cells isolated from adult male rat adrenals (Fig. 1). In these experiments, the right adrenals from each animal were pooled into an experiment for cell isolation and secretion studies, and the left adrenals were pooled and processed for PBR binding analyses (Table 1). Mitochondria collected from neonates demonstrated significantly (P < 0.003) lower PK11195 binding ability and slightly higher affinity for the ligand (Table 1). Results from the three experiments (on pd9, 10, and 11, respectively) were pooled for statistical analyses; interestingly, however, even within this small window of time there was a trend toward increasing binding ability on each day (11.5%, 12.6%, and 17.6% of adult binding, respectively).

To determine if differences in ACTH sensitivity also occurred in vivo, and to examine other neonatal ages, animals of different ages were injected once with a dose of ACTH sufficient to elevate circulating ACTH to high physiological levels. We chose to test animals on pd5, pd10, and pd15 because at these ages steroidogenesis is declining, at a nadir, and rebounding, respectively (8, 9). ACTH injection significantly increased plasma levels of ACTH in all age groups (Fig. 2). The pattern and maximal levels of ACTH achieved by the injections were statistically indistinguishable between ages 5, 10, 15, and adult, except that the response at pd10 was slightly, but significantly lower than that at pd15 (P < 0.05), but was not different from other ages. Despite the similar ACTH profiles following injection, however, the corticosterone responses to injection were significantly lower in the neonates (Fig. 2). All neonatal ages demonstrated significantly (between P < 0.001 and P < 0.05) lower corticosterone levels after ACTH injection than adults, and the response on pd10 was significantly lower than at the other two neonatal ages. Saline injection had no significant effect on plasma levels of ACTH at any age, but had small, but significant effects on plasma corticosterone at 30 min on pd15, 120 min on pd5, and 15 min in adults (Fig. 2).

In preliminary experiments, it was determined that acute ACTH injections did not significantly alter PBR expression at any age tested (not shown). Thus, mitochondria from different timepoints and treatment groups in the experiments of Fig. 2 were pooled to provide sufficient protein for use in binding studies and immunoblots. A representative Scatchard analysis of mitochondrial binding of the synthetic ligand PK11195 in neonatal and adult rats is shown in Fig. 3 and summarized in Fig. 4. Total binding capacity was higher, and affinity (Kd) of the mitochondrial receptor for PK11195 was lower, in mitochondria from adults. When the data from Fig. 4 and Table 1 were pooled, the difference in Kd between pd10 and adult rats was significant (P < 0.02).

Expression of irPBR, as evidenced by immunoblotting, was 65–80% lower in neonatal rat adrenals than in adult glands (Fig. 5). Expression of irStAR protein was highly variable and did not differ significantly between ages (Fig. 5), although it tended to be highest when PBR expression was lowest. There was also no effect of acute injections of ACTH on StAR expression at any age (not shown), although this may have been due to the slight but significant effects of saline injection on circulating ACTH and corticosterone in the animals in Fig. 2. Densitometric analysis of replicate experiments is shown in Fig. 6.

The steroidogenic responses to ACTH in Fig. 2 were quan-

![Fig. 2. Effects of saline or ACTH injection on plasma levels of corticosterone and ACTH in rats of different ages. Saline or porcine ACTH(1–39) was injected at time zero as described in Materials and Methods. A noninjected group of rats was killed at the start of each experiment for baseline values (zero time values). “Day” refers to postnatal day. Each point is the mean and SEM of 6 (adults) and approximately 10 (neonates) animals. The only significant difference in ACTH-induced ACTH levels, by 2-factor ANOVA, was between pd10 and 15. ACTH-induced corticosterone increases were significantly smaller at all neonatal ages when compared with the response in adults; the response on pd10 was the smallest of all ages. Saline injection had no effect on ACTH levels, but slightly and significantly increased corticosterone at 120 min on pd5, 30 min on pd15, and 15 min in adults.](https://academic.oup.com/endo/article-abstract/140/2/859/2990626)
tified by calculating the net area under the curve for corticosterone responses at each age group. These values were then correlated with levels of irPBR and PBR Bmax values from Figs. 4 and 6, after first correcting for nonnormality by transforming the raw data to the arcsin. The results are shown in Fig. 7. Both irPBR content and total binding capacity were highly correlated ($r^2 = 0.99$ for each) with the steroidogenic response to ACTH in vivo, and irPBR was highly correlated with PBR binding ($B_{\text{max}}$) ($r^2 = 0.99$, not shown).

Discussion

Beginning after the first few postnatal days, the rat adrenal cortex is relatively unresponsive to stimulation by ACTH and other secretagogues, reaching a nadir in responsiveness around pd10 (8, 9). At least part of the hyporesponsiveness of the immature gland may be related to reduced expression of microsomal enzymes involved in steroid synthesis (12), although all the steroidogenic enzymes, including cytochrome P450scc, are expressed in the neonatal rat adrenal at reasonably high levels when steroidogenesis is at a nadir (12). It is unlikely, therefore, that reduced expression of steroidogenic enzymes can account for all or even most of the hyporesponsiveness observed at this time. Thus, the biochemical basis of ACTH-insensitivity during the neonatal period in rats (and during fetal life in other species, Ref. 19) remains unclear.

Because previous work suggested that steps distal to generation of cAMP were the major contributors to stress- and ACTH-hyporesponsiveness of the neonatal adrenal (9, 12), we examined the ontogeny of regulatory proteins believed to be essential for cholesterol transport to occur. The process whereby free cholesterol is transported from the outer to the inner mitochondrial membrane, where P450scc is located, appears to involve several steps and at least two regulatory proteins. The first of these, the steroidogenic acute regulatory protein (StAR), is an ACTH-inducible, 30 kDa protein expressed in all steroidogenic glands except the placenta and brain (20). It appears to be required for delivery of cholesterol to contact sites between the outer and inner mitochondrial membranes (20). Once there, cholesterol may be transported through a channel created by activation of a multimeric protein complex termed the peripheral-type benzodiazepine receptor (PBR; 3, 20). PBR is an 18-kDa protein localized to the outer membrane, which in conjunction with an associated voltage-dependent anion channel, appears to complete the final step of cholesterol delivery to P450scc (3). Its importance in the steroidogenic process is highlighted by the observation that targeted disruption of this gene eliminates steroidogenesis in mouse Leydig tumor cells, an effect that is reversed upon replacement with functional PBR (4). In addition, pharmacological inhibition of PBR expression reduces circulating glucocorticoid levels and decreases adrenocortical cell sensitivity to ACTH (14).
In the present study, expression of irPBR, but not StAR, followed a developmental pattern that was highly correlated ($r^2 = 0.99$) with the developmental pattern of steroidogenesis. Immediately following birth, stress- and ACTH-induced adrenocortical steroidogenesis is relatively high (8–12, 21, 22), and then steadily declines over a period of approximately 5–6 days, reaching a nadir around pd10 (8, 9, 21, 22). By approximately pd15, ACTH-sensitivity begins to return. irPBR and PBR ligand binding capacity followed this pattern of development, and were highly correlated with steroidogenesis. StAR protein was constitutively expressed at comparable levels in neonatal and adult adrenals, and did not appear to change 2 h following ACTH injection. It is possible that the mild stressor of saline injection was sufficient to elevate resting StAR levels so as to obscure a response to ACTH. In any event, it appears from the present results that expression of PBR, not StAR, is the primary limiting factor in the ontogenic increase in steroidogenesis in rat adrenal.

The affinity of PBR for the synthetic ligand PK11195 was slightly higher in neonatal adrenal mitochondria than in adults, perhaps suggesting that the receptor is posttranslationally modified in neonates. Although we do not yet know the nature of this putative modification, the higher affinity of the neonatal receptor could conceivably contribute to the relatively high constitutive steroidogenesis that begins to characterize of neonatal rat adrenal cells (8, 9). For example, if the receptor were activated by low (basal) levels of endogenous PBR ligands, such as the endozapine diazepam binding inhibitor (3, 20), this could facilitate steroidogenesis even in the absence of ACTH. By contrast, because the total number of binding sites is reduced, maximal steroidogenesis would be expected to be only a fraction of that found in adult glands.

The physiological significance of constitutive steroidogenesis, which is unique to fetal and neonatal adrenals and is largely lost upon differentiation (8, 9), may be related to the requirement for low, but constant circulating levels of glucocorticoids during fetal/neonatal development (23). High (i.e. ACTH-induced) levels of glucocorticoids are neurotoxic during development, but constitutive levels appear to be required for proper neural and systemic differentiation (23–25). Thus, PBR may be a key regulator within the adrenal gland that allows proper titration of circulating glucocorticoids during critical windows of development. In addition, it is also possible that the relatively high expression of StAR protein on pd10 contributes to constitutive steroidogenesis. The reduced sensitivity of neonatal rat adrenals to ACTH

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Fig. 5. PBR and StAR expression in adrenal mitochondria from rats of different ages. Representative pools of mitochondria from the unstimulated rats in Fig. 2 at each of three neonatal ages or adults were loaded onto one-dimensional gels and electrophoresed and blotted as described in Materials and Methods. Bands corresponding to M, 18 kDa and 30 kDa were identified as PBR and StAR, respectively. For comparison, one lane contains mitochondrial protein isolated from unstimulated MA-10 cells (progesterone-secreting Leydig cell line). Note that PBR expression is lowest on pd10. The position of Mr markers (kilodaltons) is indicated.

Fig. 6. Densitometric analysis of replicate immunoblots for PBR and StAR proteins. Between 2–4 blots from replicate experiments, including those in Fig. 5, were analyzed as described in Materials and Methods and compared with a sample of mitochondria from adult rat adrenals on each blot (assigned a value of 100%). The results are expressed as mean and SE or range as percentage of adult value (assigned a value of 100%). *, $P < 0.001$ vs. adult.
was maintained in vitro after dispersion of adrenocortical cells, suggesting that cellular insensitivity to ACTH in vivo did not result from putative circulating inhibitory factors that interfered with ACTH action. In those experiments, adrenals used for PBR binding assays revealed a trend toward increasing activity (B max) across a limited age span from pd9–11, suggesting that this is a key window in the developmental expression of PBR. Whether PBR expression is developmentally suppressed before this time, or is induced thereafter, remains uncertain. We have previously demonstrated that chronic exposure of neonatal rats to ACTH during the hypoensive period results in accelerated maturation of the steroidogenic response to ACTH (10). Similar results have been demonstrated in fetal sheep (19). Based on the correlation between PBR and ACTH-inducible steroidogenesis in the present study, we predict that such treatment would induce a coincident increase in PBR expression and activity as well.

It is likely that proteins other than PBR follow a developmental pattern similar to the one identified in this study. For example, one of us has recently determined that cytochrome P4501B1, a xenobiotic-metabolizing enzyme, follows a very similar profile to that of PBR during neonatal ages in rats (26). Nonetheless, PBR is the only regulatory factor known to be important for ACTH-induced steroid synthesis that displays a developmental pattern similar to that of steroidogenesis. Thus, we believe that it is the expression of this important cholesterol-transporter that is the primary factor limiting steroidogenesis in the neonatal period in rats. Furthermore, the neonatal rat may provide a valuable model for determining those factors that regulate PBR expression in vivo. It should be emphasized, however, that neonatal ACTH-insensitivity is likely to be a complex phenomenon involving not only changes in cholesterol transport capacity, but changes in cytochrome P450 expression or activity, and possibly in cAMP-independent signalling pathways such as calcium/calcium-calmodulin and protein kinase C.

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