Adrenocortical Activity in the Newborn Spontaneously Hypertensive Rat
Maureen P. Malee and Ke-Ying Wu

Blood pressure is reportedly elevated in the spontaneously hypertensive rat (SHR) neonate, the etiology of which remains unclear. Aberrations in the hypothalamic-pituitary-adrenal axis have been implicated, as it is well accepted that excess corticosteroids are associated with hypertension. We examined aspects of adrenocortical activity in the neonatal SHR 1 to 21 days old and its normotensive genetic control, the Wistar-Kyoto rat (WKY). We found a fourfold greater abundance of P450scc mRNA in adrenals of SHR versus WKY day 1 neonates, and increasing but comparable abundance of adrenal P450c11B mRNA on neonatal days 1 to 21. The pattern of P450c11AS mRNA expression was distinctly different in the adrenals of SHR and WKY neonates; the relative abundance of this mRNA in SHR increased 15-fold over the 21-day period examined, whereas that in WKY remained fairly stable. RT-PCR for the presence/abundance of adrenal P450c11B3 mRNA showed absence in day 1 SHR and WKY, comparable abundances on neonatal days 7 and 14, and a distinctly greater abundance in the day 21 SHR adrenals. Peripheral corticosterone levels were threefold greater in the day 1 SHR neonate; aldosterone levels were elevated in both the SHR and WKY day 1 neonate. Thereafter, corticosterone and aldosterone levels were comparable on days 7, 14, and 21, although the anticipated depression in circulating corticosterone levels typical of the stress hyporesponsive period was noted in both SHR and WKY neonates. Although patterns of adrenocortical activity differ in the newborn SHR and WKY rat, our findings do not support an etiologic role for corticosteroids in the reported hypertension of the SHR. However, observed differences in corticosteroid profiles may augment or have a permissive effect upon the etiologic factor(s).


KEY WORDS: Hypertension, neonate, adrenal cortex, P450c11.

The spontaneously hypertensive rat (SHR) provides an experimental animal model for the study of human essential hypertension. Multiple factors are implicated in the pathogenesis of this genetic hypertension. These include a role for the sympathoadrenal system at the level of vessel reactivity to adrenergic stimulation. Components of the renin-angiotensin system (RAS) have been well studied in the adult, both circulating concentrations and at the level of gene expression; the results,

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however, do not support a convincing etiologic role.\textsuperscript{2–4} As corticosteroid excess is associated with hypertension, defects in the adult hypothalamo-pituitary-adrenal axis (HPAA) have also been investigated.\textsuperscript{5,6} Suzuki et al\textsuperscript{7} suggested that glucocorticoid enhancement of arteriolar tone in adult SHR may be involved in the observed hypertension, but treatment with a glucocorticoid antagonist had no effect on SHR blood pressure.\textsuperscript{8} Less well investigated are aspects of the HPAA in the newborn, which is particularly important in the case of SHR, as diastolic hypertension distinguishes the SHR from the WKY neonate.\textsuperscript{9,10}

The newborn period is also noteworthy for the so-called stress-hyporesponsive period (SHRP), an interval (neonatal days 2 to 14) of relative endocrine quiescence, during which time a stress-specific response is typically characterized by reduced, if any, increases in plasma corticosterone and ACTH.\textsuperscript{11} Although incompletely understood, this period may also be related to levels of corticosterone binding globulin and occupancy of glucocorticoid receptors,\textsuperscript{12} or discrepancies in levels and activities of key enzymes.\textsuperscript{13} The SHRP has not been examined in the SHR neonate. Given the potential for corticosteroid participation in the genesis of neonatal hypertension and the intriguing phenomenon of the SHRP, we characterized several aspects of adrenocortical activity in the neonatal SHR and its normotensive genetic control, the Wistar-Kyoto (WKY) rat.

METHODS

Animal Model All experiments used age-matched SHR and WKY timed-pregnant (day 14 gestation) rats, purchased from the same vendor (Harlan, Sprague-Dawley, Indianapolis, IN). They were housed under standard conditions, caged separately under constant temperature (21.5°C) with illumination from 06:00 to 18:00 and ad libitum access to commercial rat chow and water. They were left undisturbed, with the exception of animal care provision, for at least 1 week before study. On day 22, offspring delivered spontaneously. Litter size was standardized to n = 8, the number of males equal to the number of females, and neonates remaining with their dam under the conditions above specified, until death. Between 06:00 and 08:00 on neonatal days 1, 7, 14, and 21, offspring were subjected to CO\textsubscript{2} narcosis and rapidly decapitated. Trunk blood was collected and pooled as appropriate for littermates. Adrenal glands were removed and frozen immediately in liquid nitrogen for storage at −80°C.

RNA Preparation Total RNA was extracted from 2 to 6 neonatal rat adrenals by the method of Chomczynski and Sacchi\textsuperscript{14} using RNAzol (Teltest, Friendswood, TX). Adrenal tissue was sonicated in 2 mL of RNAzol. Chloroform was added (200 μL/mL) to the solution, shaken for 60 sec, and incubated (4°C) for 5 min. Samples were centrifuged (14,000 rpm; 4°C) for 30 min. The aqueous phase was removed and precipitated with an equal volume of isopropanol for 15 min at 4°C. Samples were again centrifuged (14,000 rpm; 4°C) and washed with 80% ethanol. RNA pellets were dissolved in TE buffer (pH 7.5), quantified spectrophotometrically at 260 nm and 280 nm (Gene Quant RNA/DNA Calculator, Pharmacia and UpJohn, Kalamazoo, MI), aliquoted and stored at −80°C.

Probes cDNAs for CYP11A1 (P450scc), CYP11B1 (P450c11β), CYP11B2 (P450c11AS), and β-actin were graciously provided by Drs. Synthia Mellon and Walter Miller at UCSF. Probes were single-stranded riboprobes labeled with 32P-UTP prepared from specific cDNA by transcription of linearized pKS plasmids using T3 or T7 RNA polymerase (Promega, Madison, WI). Briefly, in vitro transcription reactions of 20 μL vol contained 1X transcription buffer, 1 μg template, 500 μmol/L each ATP, GTP, CTP, 10 mmol/L DTT, 50 μCi 32P-UTP (3000Ci/mmol, Dupont NEN, Boston, MA), 20 μm Rnasin, and 15 to 20 μm T3 or T7 RNA polymerase. After incubation (37°C) for 60 min, DNA template was digested (37°C) with DNase for 20 min. Yeast transfer (t)RNA (50 μg) was added, followed by phenol:chloroform extraction and ethanol precipitation. The cRNA probe was dissolved in 50 μL TE (pH7.5) and stored at −20°C.

RNase Protection Assay RNase protection assays were performed using the RPA II kit (Ambion, Austin, TX) according to the manufacturer’s protocol, with minor modifications. Typically, 5 μg of total RNA was hybridized with 6 to 8 × 10\textsuperscript{5} cpm of cRNA probe plus 1 × 10\textsuperscript{6} cpm of rat actin cRNA probe for 12 h at 45°C. cRNA:RNA hybrids were digested with 2 μg RNaseA/40 μgRNAse T1 for 30 min at 37°C. Protected hybrids were analyzed on 5% acrylamide, 7.5 mol/L urea sequencing gel. The gel was autoradiographed overnight at −80°C. 32P-end-labeled MspI-digested pBR322 DNA fragments served as the molecular weight marker. The relative abundance of protected mRNA corresponding to the bands on the film was quantitated by scanning densitometry with the National Institutes of Health (NIH)-image 159 program, generating arbitrary units for comparisons. Assays were repeated 2 to 3 times using different samples, with the average presented for discussion.

Reverse transcriptase/PCR Amplification of Adrenal RNA cDNA was synthesized from adrenal RNA by the SuperScript Preamplification System (Gibco BRL, New York, NY) for first strand DNA synthesis according to the manufacturer’s instructions, with minor modifications. Briefly, 5 μg of total RNA was hybrid-
**TABLE 1. PLASMA LEVELS OF CORTICOSTEROIDS IN THE NEWBORN DAY 1–21 SHR AND WKY RAT**

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>cort</td>
<td>69 ± 4*</td>
<td>7 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>aldol</td>
<td>249 ± 5</td>
<td>87 ± 3</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>SHR</td>
<td>cort</td>
<td>214 ± 8</td>
<td>7 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>aldol</td>
<td>240 ± 4</td>
<td>81 ± 3</td>
<td>84 ± 3</td>
</tr>
</tbody>
</table>

*Mean ± SEM on neonatal day 1 to 21.

cort, corticosterone (ng/mL); aldol, aldosterone (pg/mL).

See text for analyses.

newborn SHR and WKY rats. Aldosterone levels are elevated on neonatal day 1 in both SHR and WKY rats; corticosterone levels are threefold higher in SHR than in WKY rats. Concentrations of corticosterone and aldosterone significantly decrease in both SHR and WKY on days 7 and 14. On day 21, aldosterone and corticosterone levels are similar in SHR and WKY rats; however, aldosterone values are greater, and corticosterone values are less than reported adult values. For corticosterone, a significant difference between groups was found (F = 193.98; Df = 1,14 P < .001), and the overall effect of time was also significant (F = 803.31; Df = 3,42 P < .001). Aldosterone levels did not vary significantly as a function of group membership and time; there was no significant interaction (F = 0.537; Df = 3,42 P < .660).

**RNase Protection Assays**

P450sc mRNA encodes the mitochondrial side-chain cleavage enzyme, which carries out the first committed step in steroidogenesis. Results (Figure 1, a and b) demonstrate a near fourfold greater abundance in neonatal day 1 SHR adrenals compared with the WKY adrenal. Thereafter, the relative abundance of this mRNA is fairly comparable over the remainder of the newborn period examined.

P450c11B encodes the enzyme responsible for the production of corticosterone. Figure 2 (a and b) illustrate that the relative abundance of this mRNA is low in the day 1 SHR and WKY neonate; thereafter, the relative abundance essentially doubles and remains similar in SHR and WKY neonatal adrenals on days 7, 14, and 21.

Aldosterone production is the consequence of 11-hydroxylase, 18-hydroxylase, and 18-oxidase activities encoded by P450c11AS mRNA. Figures 3a and 3b demonstrates that there is a 10-fold difference in the relative abundance of this mRNA in the adrenal of the WKY and SHR on neonatal day 1. Thereafter, there are 30% differences in the abundances on neonatal days 7, 14, and 21.

**RT-PCR Amplification of Adrenal RNA**

P450c11B3 encodes an enzyme with activities intermediate between P450c11B and P450c11AS. In the Sprague-Dawley neonate, it is responsible for the synthesis of an intermediate with mineralocorticoid activity.17 Figure 4 reveals the absence of this RNA in day 1 neonates, its presence on days 7 and 14, and a relatively greater abundance of the RNA in the adrenal gland of the day 21 SHR neonate.

**DISCUSSION**

The transition from intrauterine homeostasis is a dramatic one for the neonate. The neonatal period is characterized by progress toward maternal independence, with weaning, as well as by the stress-hyporesponsive period (SHRP), an interval during which the
neonate’s response to stress, as manifested by plasma ACTH and corticosterone, is stressor-dependent but typically depressed. In the neonatal SHR, reported hypertension may confound both the SHR and successful weaning if alterations in adrenocortical activity participate or are etiologic in the hypertension.

In the adult, the regulation of glucocorticoid production is primarily achieved by ACTH, whereas that of aldosterone is multifactorial. As regulation is often displayed at the level of gene expression and is manifested by discrepancies in peripheral hormone levels, we examined aspects of the adrenocortical profile of days 1 to 21 neonatal SHR and in their normotensive genetic control, the WKY rat.

An essential component in regulated steroidogenesis is cytoplasmic cholesterol translocation from the outer to the inner mitochondrial membrane. A mitochondrial phosphoprotein, steroidogenic acute regulatory protein (StAR), is essential for this translocation, and its synthesis is induced by ACTH. StAR presence and regulation have not been investigated in the neonate. Mellon et al have described adrenocortical gene expression in the Sprague-Dawley (SD) neonatal rat. Cholesterol conversion is initiated by P450ccc, or side chain cleavage enzyme, which is encoded by the CYP11A1 gene. This enzyme is broadly distributed throughout the entire cortex (glomerulosa, fasciculata/reticularis), as are the other enzymes involved in the synthesis of 11-deoxycorticosterone (DOC). Zone-specific expression characterizes the two enzymes, P450c11B and P450c11AS. The CYP11B1 gene encodes P450c11B, which is expressed in the fasciculata/reticularis, and encodes 11-hydroxylase, which converts DOC to corticosterone or 18-OH-DOC. The CYP11B2 gene, which encodes P450c11AS, is expressed in the glomerulosa and encodes aldosterone synthase (11-hydroxylase, 18-hydroxylase, and 18-oxidase), which converts DOC to aldosterone. The CYP11B3 gene, or P450c11B3 mRNA, has a zonal distribution similar to P450c11B mRNA. It is present for a limited interval in the Sprague-Dawley neonate. The enzymatic activity of its protein appears to catalyze the conversion of DOC to 18-OH-DOC, corticosterone, and 18-OH-corticosterone. Reports addressing gene expression beyond message abundance are few in number. Nagaya et al reported comparable immunoreactive levels of mitochondrial P450ccc and c11B proteins on neonatal days 1 and 10, noting, however,
that protein levels do not necessarily correlate with enzyme activity in the SD neonatal rat. This dissociation also extends to message abundance and peripheral hormone levels in the fetal and adult SD rat as reported by others.\textsuperscript{18,19} The patterns of adrenocortical activity in neonatal SHR and WKY rats differ considerably, and differ from that in the SD neonate. There is evidence of dissociation of message abundance and peripheral hormone levels as well. We found a near fourfold greater abundance of P450scc mRNA and a conspicuously low abundance of P450c11AS mRNA in the adrenal of the SHR day 1 neonate, a time during which blood pressure in SHR neonates is significantly greater in SHR versus WKY rats.\textsuperscript{9,10} The abundance of P450c11B mRNA in SHR and WKY was comparable. Aldosterone levels were increased in SHR and WKY, as is typical in the day 1 neonate;\textsuperscript{20} corticosterone levels are comparable. Others have also noted the dissociation of message abundance and peripheral hormone levels in fetal and adult SD rats.\textsuperscript{18,19} Nagaya et al noted that immunoreactive protein levels do not necessarily correlate with enzyme activity in the SD neonatal rat.\textsuperscript{13} Moreover, we did not examine the stability or rate of transcription of P450scc, P450c11B, or P450c11AS mRNAs, which may also account for discrepancies in relative mRNA abundances.

Factor(s) responsible for the regulation of gene expression and peripheral hormone levels in the day 1 neonate are unclear. The HPA is thought to be intact and functional in the late gestation SD rat, and renal renin-angiotensin system (RAS) development/functional maturation continues in the neonatal period. Although reported in the older SHR and WKY neonate and found to be comparable,\textsuperscript{21} pituitary and circulating levels of ACTH have not been reported in the day 1 neonate. The renal and adrenal RAS may participate in the regulation of aldosterone, although information beyond a higher renal renin activity in day 1 neonatal SHR is lacking.\textsuperscript{22} The degree to which the HPA is involved in the regulation of both corticosterone and aldosterone, as well as message expression, remains unclear. Perhaps an elevated ACTH and plasma renin activity combined to increase peripheral aldosterone by favoring an acceleration of aldosterone synthase enzyme activity. A decline in P450c11AS mRNA abundance with little effect on P450c11B mRNA abundance is reported in the day 7 SD neonate in response to ACTH treatment.\textsuperscript{22} Perhaps the same is the case in the day 1 neonate.
Neonatal days 7 and 14 are within the SHRP, the mechanism of which remains poorly understood. Increased neonatal sensitivity to glucocorticoid feedback, as well as increased expression of a corticotropin release-inhibiting factor, have been offered as etiologic. During the SHRP in SD, basal and stress-stimulated corticosterone and ACTH levels are very low. Corticosterone concentrations on days 7 and 14 in our study of SHR and WKY neonates were significantly depressed, as reported previously. On neonatal day 7, aldosterone levels were comparable in SHR and WKY rats, and similar to those reported in the day 7 SD rat. Furthermore, although not examined on the same neonatal days, the pattern of aldosterone reported herein is not unlike that reported by Mullins et al in SHR and WKY rats, and the levels are depressed relative to neonatal days 1 and 21. When examined on days 7 and 14, the relative abundances of adrenal P450scc, P450c11B, and P450c11AS mRNAs in SHR and WKY neonates were comparable.

During the SHRP, the regulation of circulating hormone levels and mRNA abundances is unclear. Ardekani et al reported comparable circulating and pituitary levels of ACTH in 10- and 20-day-old SHR and WKY rats. Mellon et al have reported no apparent regulation of P450scc, P450c11B, or P450c11AS mRNAs by ACTH in the neonatal Sprague-Dawley rat on day 12. This is contrary to the report of Feuillan and Aguiler, who found a striking decline in P450c11AS mRNA in response to ACTH and dexamethasone treatment, with a much lesser response of the relative abundance of P450c11B mRNA to these manipulations. They concluded that aldosterone production is very sensitive to, and dependent on, a low basal level of ACTH and that a factor other than ACTH participated in aldosterone production. The HPA may affect the production of corticosteroids at the level of translation or enzyme activity, neither of which we examined in SHR and WKY rats. Nagaya et al examined the premise that the SHRP partially results from deficiencies in steroid enzyme content. They found that the immunoreactive levels of side chain cleavage enzyme and 11-hydroxylase did not change during the SHRP. Furthermore, immunoreactive levels of 3B-hydroxysteroid dehydrogenase and 21-hydroxylase are developmentally and differentially regulated, and immunoreactive levels of these proteins are not correlated with enzyme activity, perhaps reflective of multiple isofoms of the two enzymes. P450c11B mRNA, on the other hand, appears to be negatively regulated by ACTH in day 12 and 18 neonatal Sprague-Dawley males. Taken together, the neonatal HPA is unlikely
of singular importance in the observed patterns of adrenocortical gene expression that we report in the SHR and WKY neonate.

Other potential participants in the regulation of aldosterone include the RAS. Sinaiko and Mirkin\(^{24}\) have reported that renal renin activity is consistently higher in SHR on neonatal days 1 to 21. Differences in volume, sodium and potassium balance, as well as ANP, can also influence aldosterone production. McCarty and Tong\(^{25}\) examined milk electrolytes from lactating SHR and WKY females coincident with the neonatal period reported herein. They proposed that a preweanling electrolyte discrepancy may serve as an environmental trigger for the progressive age-related increases in hypertension in SHR offspring. The impact of electrolyte discrepancies upon the reported pattern of adrenocortical activity is unclear, but remains of interest inasmuch as Tremblay and LeHoux\(^{26}\) have reported transcriptional activation of adrenocortical steroidogenic genes by altered sodium and potassium intake.

By neonatal day 21, corticosterone and aldosterone levels, as well as the abundances of P450scc, P450c11B, and P450c11AS mRNAs, are comparable. Distinctly perplexing is the increased abundance of P450c11B3 in the day 21 SHR neonatal adrenal, which can encode enzyme activity responsible for considerable production of 18-OH-DOC, a steroid with mineralocorticoid activity. This mRNA is reportedly negatively regulated by ACTH in the day 12 and 18 male Sprague-Dawley neonate.\(^{17}\) Others have shown pituitary and circulating ACTH to be comparable in the SHR and WKY neonate,\(^{21}\) and our samples were comprised of 50:50 male:female adrenals. We hypothesize that this mRNA is regulated by ACTH as well as other factor(s) in the SHR; furthermore, the 18-OH-DOC potentially produced may be a steroid that participates in the propagation of hypertension in SHR, as has been proposed in the transgenic rat model of hypertension.\(^{27}\)

In summary, the observed patterns of mRNA expression and circulating hormone levels do not otherwise support an etiologic role for adrenocortical activity in the reported hypertension in neonatal SHR. A permissive effect on etiologic factor(s) remains a possibility.

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