Neonatal hypoxic hyperlipidemia in the rat: effects on aldosterone and corticosterone synthesis in vitro

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1Endocrine Research Laboratory, St. Luke's Medical Center, Milwaukee 53215; 2Department of Medicine, Medical College of Wisconsin, Milwaukee 53226; and 3Wm. S. Middleton Memorial Veterans Hospital and the Departments of Medicine and Pharmacology, University of Wisconsin School of Medicine, Madison, Wisconsin 53705

Raff, Hershel, Eric D. Bruder, Barbara M. Jankowski, and Theodore L. Goodfriend. Neonatal hypoxic hyperlipidemia in the rat: effects on aldosterone and corticosterone synthesis in vitro. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R663–R668, 2000.—Neonatal hypoxia increases aldosterone production and plasma lipids. Because fatty acids can inhibit aldosterone synthesis, we hypothesized that increases in plasma lipids restrain aldosteronogenesis in the hypoxic neonate. We exposed rats to 7 days of hypoxia from birth to 7 days of age (suckling) or from 28 to 35 days of age (weaned at day 21). Plasma was analyzed for lipid content, and steriodogenesis was studied in dispersed whole adrenal glands untreated and treated to wash away lipids. Hypoxia increased plasma cholesterol, triglycerides, and nonesterified fatty acids in the suckling neonatal rat only. Washing away lipids increased aldosterone production in cells from 7-day-old rats exposed to hypoxia, but not in cells from normoxic 7-day-old rats or from normoxic or hypoxic 35-day-old rats. Addition of oleic or linolenic acid to washed cells inhibited both aldosterone and corticosterone production, although cells from hypoxic 7-day-old rats were less sensitive. We conclude that hypoxia induces hyperlipidemia in the suckling neonate and that elevated nonesterified fatty acids inhibit aldosteronogenesis.

adrenal cortex; steriodogenesis; fatty acids; cholesterol; triglycerides

Hypoxia is a devastating cause of morbidity and mortality in the neonate (5, 13). Although considerable attention has been directed at neurological, cardiopulmonary, and renal adaptations to hypoxia in the neonate has not been extensively evaluated. Neonatal hypoxia results in significant derangements in plasma lipids, particularly in nursing infants. This has been termed neonatal hypoxic hyperlipidemia, and studies performed over 25 years ago suggest that it may be due to direct effects on hepatic function (3, 10).

We recently demonstrated an augmentation of aldosteronogenesis in 7-day-old rats exposed to hypoxia from birth (19); this effect is the opposite of our findings in the adult rat exposed to hypoxia, in which aldosterone production was inhibited due to a decrease in P450c11AS activity and mRNA expression (20). We noticed that plasma from suckling neonatal rats exposed to hypoxia from birth was very lipemic. We have shown that fatty acids inhibit aldosteronogenesis in adrenal cells from hypoxic nursing rabbits (21). The current study was designed to examine the role of altered lipids on aldosteronogenesis in our model of neonatal hypoxia in the rat. In particular, we hoped to reconcile the augmentation of aldosteronogenesis we observed in neonatal hypoxic rat pups (19) with the hyperlipidemia that would be expected to inhibit aldosteronogenesis (7–9, 21).

The aims of the present study were to 1) analyze changes in plasma lipids in neonatal, suckling rats exposed to hypoxia for 7 days from birth compared with juvenile, weaned rats exposed to hypoxia for 7 days (from 28 to 35 days of age), 2) determine the effect of removing nonesterified fatty acids (NEFAs) on adrenocortical steriodogenesis in vitro, and 3) determine the effect of adding back fatty acids on steriodogenesis in washed adrenal cells.

METHODS

Animal treatment. All experimentation was approved by the Institutional Animal Care and Use Committees of the Medical College of Wisconsin and St. Luke's/Sinai Samaritan Medical Center. Timed-pregnant Sprague-Dawley rats (Harlan Sprague Dawley) were obtained at 14 days gestation and maintained on a standard sodium diet (Richmond Standard 5001) and water ad libitum in a controlled environment (0600–1800 lights on). Parturition usually occurred on the afternoon of gestational day 21, during which rats were kept under observation.

Hypoxia from 0 to 7 days of age. As soon as a litter was completely delivered, the dam and her pups [40 litters; 8–10 pups (male and female)/litter] were immediately moved to an environment chamber and exposed to normobaric normoxia (21% O2; room air) or hypoxia (12% O2) as described previously (18, 22, 25). Each experimental day was at the end of 7 days of exposure of four dams and their litters to either normoxia or hypoxia. We previously showed that this exposure leads to arterial PO2 levels in adults of ~50–55 Torr with sustained hypocapnia and alkalosis (18, 22). Lactating dams were maintained with their litters for 7 days in a normoxic or hypoxic environment (19, 25). Chambers were briefly opened on day 4 to clean the cages. At 0800 on day 7, rat pups were
decapitated and trunk blood was collected for the measurement of plasma total cholesterol, triglycerides, and total NEFAs. Each litter averaged ~8–10 pups and, therefore, ~32–40 pups were used to generate adrenal cells for each “experimental day” (batch of cells). Blood from three pups was pooled and treated as one sample. Adrenal glands were quickly removed. Lactating dams remained in normoxia or hypoxia for 3–5 h, anesthetized with sodium methohexital (30 mg), given 5 U ip oxytocin, and then milk was expressed as described previously (26).

Hypoxia from 28 to 35 days of age. Male and female rats (n = 133) from randomly assigned litters raised under normoxic conditions were weaned at 21 days of age. At 28 days of age, they were placed in chambers, exposed to normoxia or hypoxia for 7 days, and trunk blood and adrenal glands were collected as described above. Approximately 16 rats (4/chamber) were used to generate adenals for each experimental day (end of 7 days of normoxia or hypoxia).

Cell preparation and experimental protocols. Whole adrenal glands were minced and dispersed with collagenase (Worthington). Although Feuillan and Aguilera (4) were able to separate the capsule (zona glomerulosa; ZG) from the subcapsule (zona fasciculata/reticularis) in 7-day-old rat adrenals, we were unable to get a clean separation of zones in hypoxic 7-day-old rats to generate sufficient capsular (ZG) cells to perform the experiments described. Therefore, whole adrenal glands were used in all experimentation. Approximately 80 adrenals from 7-day-old rats and 32 adrenals from 35-day-old rats were used to generate each batch of cells (experimental day). The dispersed cells were washed, counted by hemocytometry and trypsin blue exclusion, and placed in cold Krebs-HEPES-calcium buffer at a concentration of 50,000 cells/ml. Final experimental buffer contained either 0.1% fatty acid-free albumin (Sigma “essentially fatty acid-free” albumin; ≥97% pure) or no albumin. Cells were always studied the day they were dispersed. Cells were placed in test tubes and incubated for 2 h at 37°C in a shaking water bath as described previously (20). Each treatment within an experimental day (basal, cAMP, ACTH) was performed in triplicate, averaged, and treated as one value. Each “experimental day” (batch of cells), the basal and steroidogenic responses to dibutyryl cAMP (3 mM) and synthetic rat ACTH (Peninsula Labs, 20 ng/ml) in adrenal cells from one of the following were analyzed: normoxic 7-day-old rats, hypoxic 7-day-old rats, normoxic 35-day-old rats, and hypoxic 35-day-old rats. n Values on figures represent the numbers of different cell preparations (experimental days) for each of these exposures/ages of rats.

In the first set of experiments, cells from normoxic or hypoxic pups were incubated under normal conditions (unwashed) or free of NEFAs (washed) as described previously (21). Briefly, cells were preincubated in buffer without cAMP or ACTH stimulation containing 0.1% fatty acid-free albumin for 50 min at 37°C, washed twice, then studied in buffer containing 0.1% fatty acid-free albumin. Unwashed cells were preincubated without cAMP or ACTH in buffer with 0.1% normal bovine serum albumin (Sigma ≥97% purity) for 50 min and then resuspended in buffer containing 0.1% fatty acid-free albumin for experimentation (basal, cAMP, or ACTH stimulation for 2 h).

In the second set of experiments, the effects of oleic and linolenic acid on aldosterone release were evaluated as described previously (21). Cells were washed in buffer containing 0.1% fatty acid-free albumin as described above and then suspended in albumin-free buffer. Washed cells from 7-day-old rats were studied without stimulation (basal) and with cAMP or ACTH stimulation as described above in the presence of oleic acid (0, 5, and 50 µM; Sigma) for 2 h at 37°C. Fatty acids were added to cell suspensions from a stock solution, giving a final ethanol concentration of 0.2%. The effects of linolenic acid on basal and cAMP-stimulated aldosterone production were assessed in washed cells from 7-day-old rats. Finally, the effects of oleic and linolenic acid on cAMP-stimulated steroidogenesis in washed cells from 35-day-old rats were assessed.

Measurements. Total plasma cholesterol was measured after hydrolysis of esters by cholesterol esterase and oxidation by cholesterol oxidase to cholest-4-en-3-one and H2O2. H2O2 was measured by spectrophotometry after addition of chromogen (Sigma Diagnostics Procedure 352). Plasma triglycerides were measured by hydrolysis with lipoprotein lipase to glycerol and free fatty acids followed by measurement of glycerol using sequential enzymatic steps to form formazan, which was detected by spectrophotometry (Sigma Diagnostics Procedure 336). Total plasma NEFAs were measured by a radiochemical assay (21), which measures NEFA transport of radioactive nickel from the aqueous to the organic phase. The assay has a sensitivity of 10 µmol/l, recovery of ≥90%, and interassay coefficient of variation of 2%. Milk lipid content was estimated using the creamatocrit method (12). Aldosterone and corticosterone concentrations in the cell supernatant were assayed by previously described RIAs (19–21). Statistical analysis. Each batch of cells was considered one experiment and was derived from 7-day-old litters (4/batch)
Table 1. Aldosterone and corticosterone production by unwashed vs. washed adrenal cells in rats exposed to hypoxia for 7 days (0–7 days of age or 28–35 days of age)

<table>
<thead>
<tr>
<th></th>
<th>Cells from Normoxic Rats</th>
<th></th>
<th>Cells from Hypoxic Rats</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>dbcAMP</td>
<td>ACTH</td>
<td>Basal</td>
</tr>
<tr>
<td>7 Day old</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unwashed</td>
<td>5.1 ± 1.5</td>
<td>14.4 ± 3.9*</td>
<td>7.7 ± 1.7*</td>
<td>7.3 ± 4.0</td>
</tr>
<tr>
<td>Washed</td>
<td>5.7 ± 1.8</td>
<td>20.2 ± 6.5*</td>
<td>11.1 ± 1.6*</td>
<td>14.4 ± 4.4†</td>
</tr>
<tr>
<td>35 Day old</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unwashed</td>
<td>2.0 ± 1.1</td>
<td>16.7 ± 4.6*</td>
<td>5.6 ± 1.5*</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>Washed</td>
<td>2.4 ± 1.0</td>
<td>27.2 ± 11.2*</td>
<td>7.1 ± 2.8*</td>
<td>2.7 ± 1.1</td>
</tr>
</tbody>
</table>

Corticosterone

|                |                         |                       |                         |
| 7 Day old      |                          |                       |                         |
| Unwashed       | 83 ± 36                 | 296 ± 33*             | 155 ± 28*               | 149 ± 50              | 699 ± 280*            | 291 ± 105*             |
| Washed         | 89 ± 19                 | 206 ± 35*             | 128 ± 24*               | 219 ± 41              | 654 ± 136*            | 442 ± 95*              |
| 35 Day old     |                          |                       |                         |
| Unwashed       | 61 ± 22                 | 1,557 ± 978*          | 259 ± 95*               | 91 ± 30               | 954 ± 71*             | 207 ± 50*              |
| Washed         | 96 ± 60                 | 1,037 ± 418*          | 415 ± 232*              | 82 ± 17               | 706 ± 244*            | 194 ± 43*              |

Values are means ± SE in ng·10⁶ cells⁻¹·h⁻¹. dbcAMP, dibutyryl cAMP. *Indicates different from basal; † indicates different from unwashed with same treatment.

and 35-day-old rats (∼15/batch). Replications of each condition within an experiment were averaged and treated as one datum. Data were analyzed by two-factor ANOVA repeated on both factors and Duncan’s multiple-range test. Because of significant variation of basal and maximally stimulated steroidogenesis between cell preparations, some of the data were normalized to basal secretion of unwashed cells for each batch of cells (experimental day). For analysis of normalized data, Friedman nonparametric test was used. Because of the variability in steroidogenesis between batches of cells, the statistical analysis focused on the effects (i.e., unwashed vs. washed, and maximal steroidogenesis between different cell preparations (Table 1) and for clarity of presentation, data presented in Fig. 2 are normalized to basal aldosteronogenesis in unwashed cells. Washing adrenocortical cells and incubating in buffer with fatty acid-free albumin did not result in a significant change in aldosteronogenesis in cells from normoxic 7-day-old rat pups. By contrast, washing and incubating cells in buffer with fatty acid-free albumin resulted in a marked increase in basal, cAMP-stimulated, and ACTH-stimulated aldosteronogenesis by cells from hypoxic 7-day-old rats. There was no significant effect of washing on aldosterone production by cells from normoxic or hypoxic 35-day-old rats. Table 1 and Fig. 3 demonstrate that there was no effect of washing and incubating in fatty acid-free albumin on corticosterone production in cells from 7- or 35-day-old normoxic or hypoxic rats. The corticosterone response to cAMP and ACTH ap-

RESULTS

Figure 1 shows the plasma lipid levels from rat pups exposed to hypoxia from birth to 7 days of age and from juvenile rats weaned at 21 days of age and subsequently exposed to hypoxia from 28–35 days of age. Hypoxia from birth to 7 days of age resulted in significant increases in total cholesterol, triglycerides, and NEFAs. There was no effect of hypoxia on total cholesterol, triglycerides, or NEFAs in 35-day-old rats exposed to hypoxia for 7 days. Although all lipid levels tended to be lower in 35-day-old normoxic compared with 7-day-old normoxic rats, the only statistically significant difference was in triglycerides.

Total fat content was significantly higher in milk expressed from hypoxic dams (17.6 ± 1.1%; n = 11) vs. normoxic dams (12.3 ± 1.4%; n = 11) dams.

Table 1 and Fig. 2 show aldosterone production in vitro by washed and unwashed cells from normoxic and hypoxic rats. Because of significant variation of basal and maximal steroidogenesis between different cell preparations (Table 1) and for clarity of presentation, data presented in Fig. 2 are normalized to basal aldosteronogenesis in unwashed cells. Washing adrenocortical cells and incubating in buffer with fatty acid-free albumin did not result in a significant change in aldosteronogenesis in cells from normoxic 7-day-old rat pups. By contrast, washing and incubating cells in buffer with fatty acid-free albumin resulted in a marked increase in basal, cAMP-stimulated, and ACTH-stimulated aldosteronogenesis by cells from hypoxic 7-day-old rats. There was no significant effect of washing on aldosterone production by cells from normoxic or hypoxic 35-day-old rats. Table 1 and Fig. 3 demonstrate that there was no effect of washing and incubating in fatty acid-free albumin on corticosterone production in cells from 7- or 35-day-old normoxic or hypoxic rats. The corticosterone response to cAMP and ACTH ap-

Fig. 2. Aldosterone production in cells from normoxic and hypoxic 7 (A)- and 35 (B)-day-old rats. Cells were unwashed or studied after washing away lipids and incubating in buffer containing fatty acid-free albumin. Data are presented as fold increase over basal unstimulated state within an experimental day (either normoxia or hypoxia). *Significant difference from basal; † washed different from unwashed.
peared to be more robust in cells from 35-day-old vs.
7-day-old rats, particularly from normoxic rats.

Figure 4 shows the effect of oleic acid on aldosterone
and corticosterone production in washed cells from
7-day-old normoxic and hypoxic rats. In all cases,
addition of oleic acid inhibited aldosterone synthesis.
The data show that aldosteronogenesis by washed cells
from hypoxic rats was less sensitive to inhibition by 5
µM oleic acid than cells from normoxic rats. In fact, 5
µM oleic acid attenuated the aldosterone response to
ACTH in cells from 7-day-old normoxic rats, whereas, if
anything, it slightly augmented the response in cells
from hypoxic 7-day-old rats. The results with linolenic
acid were similar to oleic acid (data not shown). The
effect of oleic acid on corticosteronogenesis was similar
to that for aldosteronogenesis, except that ACTH-
stimulated corticosterone production was not inhibited
by 5 µM oleic acid (Fig. 4). The effect of linolenic acid on
corticosteronogenesis was similar to oleic acid (data not
shown). Figure 5 shows the effect of addition of oleic
acid on cAMP-stimulated steroidogenesis in washed
cells from normoxic and hypoxic 35-day-old rats; high
concentrations of fatty acids inhibited steroidogenesis
in all cases.

**DISCUSSION**

This study evaluated the effect of 7 days of hypoxia
on plasma lipids and adrenocortical steroidogenesis in
neonatal (suckling) and juvenile (weaned) rats. Hyp-
oxia induced significant hypercholesterolemia, hypertri-
glyceridemia, and elevated fatty acids in suckling but
not weaned rats. The total fat content of milk from
hypoxic dams was ~50% higher than milk from nor-
moxic dams. Washing and incubating adrenocortical
cells and incubation in buffer with fatty acid-free
albumin augmented aldosteronogenesis but not cortico-
steronogenesis, and this effect was observed only in
hypoxic neonatal (suckling) rats. Adding either oleic or
linolenic acid inhibited steroidogenesis. These results
suggest that hyperlipidemia restrains steroidogenesis
in the suckling hypoxic neonate. The increase in aldoste-
ronogenesis we previously demonstrated in 7-day-old
hypoxic rats occurred despite the inhibitory influence of
high lipids (19, 21).

These studies confirm older publications demonstrat-
ing neonatal hypoxic hyperlipidemia (3, 10). The hypox-
ia-induced increases in total plasma cholesterol, triglyc-
erides, and NEFAs occurred only in suckling rats. This
phenomenon has been attributed to hepatic dysfunc-
tion (3), and may also reflect an increase in the total fat
content of ingested milk reported herein. Thirty-five-
day-old rats eating normal rat chow (which is relatively
low in lipid content) did not display hyperlipidemia
when exposed to hypoxia.

The hypoxic neonate presents an interesting para-
dox: an increase in aldosteronogenesis and plasma
aldosterone (19) in the face of high levels of inhibitory
lipids. At this point, we cannot resolve the mechanisms
of this effect. The relative insensitivity of cells from

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**Fig. 3. Corticosterone production in same experiments as in Fig. 2. A,
7-day-old rats; B, 35-day-old rats. * Significant difference from basal.**

**Fig. 4. Effect of oleic acid on basal, cAMP-, and ACTH-stimulated
aldosterone and corticosterone production in washed cells from
7-day-old normoxic (A) and hypoxic (B) rats. * and † Significant
difference from 0 µM oleic acid; † different from basal at same
concentration of oleic acid.**
hypoxic 7-day-old rats to inhibition by oleic and linolenic acid may partly explain this finding. It is also possible that fatty acids and other lipids were so elevated in 7-day-old hypoxic (nursing) pups that washing was not complete, so lower concentrations of exogenous fatty acids had no effect.

Cells from normoxic vs. hypoxic rats were prepared on different experimental days, and the variability between them was relatively large compared with our previous study in which cells from normoxic and hypoxic neonates were prepared on the same day (19). This minimized our ability to compare absolute steroidogenesis in cells from normoxic vs. hypoxic rats with great power or confidence as we did previously (19). It is important to note that the number of experiments within a treatment in the present study (n = 4 or 5), although small, is similar or greater than our previous study (19). This suggests that the variability could be partially due to different batches of cells and that more experiments would not have greatly reduced this variability. There are trends in Table 1 (nonnormalized data) not readily evident from the normalized data in Figs 1–5. The differences in aldosterone production rates between cells from normoxic and hypoxic rats were fairly similar regardless of age. As noted above, the augmenting effect of washing away lipids was only observed in cells from hypoxic 7-day-old rats, and this finding held whether data were normalized or not.

The most striking differences in Table 1 appeared to be in corticosterone production. Although this study was not specifically designed to examine this phenomenon, our data suggest that stimulated corticosterone production was greater in cells from 35- vs. 7-day-old normoxic rats. This phenomenon of relatively decreased steroidogenic capacity in neonatal rats has also been demonstrated previously (2, 16). It also appeared that cells from hypoxic 7-day-old rats had higher corticosterone responses to cAMP. As stated above, the variability between batches of cells made detection of significant differences impossible. Although washing cells from hypoxic rat pups had no effect on corticosterone production, addition of high concentrations of fatty acids to washed cells decreased basal, cAMP-stimulated, and ACTH-stimulated corticosterone production. This may be a nonspecific effect. There may be significant species differences in the effects of fatty acids because cortisol production from bovine cells is relatively insensitive to inhibition by addition of fatty acids (8, 9), whereas fatty acids inhibit corticosterone production from rat adrenocortical cells (14).

Several lines of evidence showing differences in the regulation of aldosterone production in the neonatal vs. adult rat help to explain the results of the current study. Aldosterone production is relatively more ACTH dependent in the human and rat neonate compared with the adult (4, 23, 24). Furthermore, we previously demonstrated that plasma renin activity was not increased by hypoxia in the 7-day-old rat when plasma aldosterone (and ACTH) levels were very high. Fatty acids may contribute to the isolation of the ZG from angiotensin, because binding of angiotensin to its receptors is inhibited by low concentrations of fatty acids (7, 9). A recent study also suggested that angiotensin AT1 receptor mRNA levels are relatively low in the rat adrenal cortex until after 25 days of age (15).

Perspectives

We have documented dramatic hyperlipidemia and increased plasma NEFAs in the suckling neonate exposed to hypoxia. This phenomenon has received very little attention despite its potential clinical importance and considering that fatty acids are very important regulators of a wide variety of physiological and hormonal systems (27). The maintenance of aldosterogenesis is an important component of the adaptation to hypoxia in the neonate. It is now accepted that, in the adult of many species, inhibition of aldosterogenesis is an integral component of the adaptation to hypoxia, because it allows natriuresis and diuresis and helps prevent life-threatening pulmonary and cerebral edema (11, 17). However, sodium and fluid balance in the neonate is considerably more tenuous. Maintenance or increases of fluid and blood volume are definite assets under hypoxic conditions in the neonate (1, 13, 24). The current experiments suggest that the augmentation of aldosterogenesis we have previously demonstrated in the 7-day-old hypoxic rat occurs despite inhibition by high plasma lipids. The ZG is less sensitive to inhibition by fatty acids, allowing continued augmented aldosterogenesis in the hypoxic neonate.

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