Oxygen-dependence of ACTH-stimulated aldosterone and corticosterone synthesis in the rat adrenal cortex: developmental aspects

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Abstract

The control of ACTH-stimulated steroidogenesis under decreasing levels of O₂ is not fully understood. The purpose of this study was to examine the effects of decreased O₂ *in vitro* on rat adrenocortical steroid synthesis at different stages of development. Of interest was the evaluation of the effect of low O₂ on steroidogenesis during the stress hyporesponsive period of the neonate. Rats were killed at 7, 14, or 42 days of age, adrenals collected and capsules (zona glomerulosa, ZG) separated from subcapsules (zona fasciculata/reticularis, ZFR). Cells were dispersed and placed into glass vials each gassed with a different level of O₂ (21, 5, 2, 1, or 0% O₂). The entire steroidogenic pathway was analyzed by measuring ACTH-stimulated cAMP, corticosterone and aldosterone production during a 2 h incubation. In addition, the early (P450 scc) and late (P450c11 β and P450 ald) pathway activities were examined in the presence of cyanoketone. The PO₂ for half-maximal activity (P₅₀) for aldosterone synthesis in ZG cells from 7- and 42-day-old rats was

approximately 28 mmHg and 7 mmHg respectively, indicating that cells from older rats were more resistant to inhibition by low O₂. The P₅₀ for cAMP production from the ZG was $\sim$ 14 mmHg for both age groups. The P₅₀ for corticosterone synthesis was $\sim$ 28 mmHg and $<$ 7 mmHg in ZFR cells from 7- and 42-day-old cells respectively. The only enzyme activities affected by low O₂ ($<$ 35 mmHg) were P450 ald and P450 scc. Moderate decreases in O₂ (from $\sim$ 150 mmHg) decreased aldosteronogenesis, possibly due to observed decreases in cAMP generation, but not due to decreases in steroidogenic enzyme activity (7-day-old). Severe decreases in O₂ presumably inhibited P450 ald through a direct effect on enzyme activity (both ages). P450 scc activity (including cholesterol transport) also seems to be decreased by very low O₂ (7-day-old). These findings illustrate a novel developmental alteration in O₂-regulated steroid production, and may have implications for neonatal health and disease.

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Introduction

The adaptation to hypoxia involves an integrated response among different organ systems. The ability to adapt to hypoxic conditions depends on cardiovascular, respiratory, renal and endocrine responses (Friedman & Fahey 1993, Thomas & Marshall 1995). It is advantageous for an organism to minimize the fluid and salt retention that occurs during hypoxic exposure. This would be beneficial because it prevents the development of cerebral and pulmonary edema, conditions often observed during acute mountain sickness (Slater *et al.* 1969). A decrease in the mineralocorticoid aldosterone would lead to this desired natriuresis and diuresis. Although decreased plasma aldosterone levels have been demonstrated in a number of hypoxic models *in vivo* (Raff 1991), the exact cellular

mechanism of this decrease has yet to be elucidated across species.

Previous studies utilizing bovine adrenocortical cells *in vitro* have found that hypoxia directly inhibits aldosterone synthesis (Raff *et al.* 1989). This inhibition occurred in the presence of known stimulators of aldosterone production, such as adrenocorticotrophic hormone (ACTH), 3',5'-cyclic adenosine monophosphate (cAMP), potassium, and angiotensin II (AII) (Raff *et al.* 1989, Raff & Kohandavish 1990, Brickner *et al.* 1992). Subsequent studies in bovine cells found that aldosterone production was inhibited in proportion to decreases in O₂ through inhibition of the aldosterone synthase enzyme (P450 ald) (Raff & Kohandavish 1990, Brickner *et al.* 1992).

The effects of hypoxia on adrenocortical function have been examined in rats *in vivo*. Results have revealed that

the rat zona glomerulosa (ZG) is more resistant to acute changes in O_2 *in vivo* than other species previously studied (Raff & Roarty 1998). Chronic hypoxia *in vivo* in adult rats decreases the expression of specific adrenal steroidogenic enzymes (Raff *et al.* 1996). Furthermore, the rat adrenocortical response to hypoxia *in vivo* exhibits differences at various stages of development (Raff *et al.* 1996, Raff *et al.* 1999). These differences are the result of a period of relative insensitivity to stressful stimuli at the level of the adrenal cortex. Studies have shown that this stress hyporesponsive period occurs during the first three weeks of life, and that the magnitude and patterns of steroidogenesis vary with developmental age and are also stressor-specific (Walker *et al.* 1991). It appears that diminished ACTH and corticosterone responses during this period are not due to hypothalamic dysfunction; rather, they are due to decreased sensitivity to stimuli at the level of the pituitary and adrenal (Walker *et al.* 1986). Therefore, it would be of interest to examine O_2 -dependent adrenal steroidogenesis *in vitro* during this unique period of development.

The purpose of this study was to assess the effects of hypoxia on ACTH-stimulated steroidogenesis *in vitro*. Using an *in vitro* model allowed us to study aldosterone production in the absence of known inhibitory factors such as atrial natriuretic peptide (ANP) (Elliot & Goodfriend 1986). We chose to study ACTH-stimulated steroid production because previous studies have shown that AII is not the primary controller of aldosterone synthesis in the 7-day-old rat (Feuillan & Aguilera 1996). Our first aim was to determine whether rat adrenocortical cells studied *in vitro* would display a sensitivity to low O_2 similar to that previously shown in bovine cells. The second aim of our research was to evaluate developmental differences in the adrenocortical response to hypoxia. This was carried out using cells from neonatal rats and from juvenile, weaned rats. The final aim of the study was to assess isolated components of the steroidogenic enzyme pathway during hypoxia *in vitro* in cells from rats of different ages.

Materials and Methods

Cell preparation

All experimentation was approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin and St Luke's/Sinai Samaritan Medical Center. Timed-pregnant Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA; $n=42$) were obtained at 14 days gestation and maintained on a standard sodium diet (Richmond Standard 5001, Brentwood, MO, USA) and water *ad libitum* in a controlled environment (lights on, 0600–1800 h). Parturition usually occurred on the afternoon of gestational day 21.

Lactating dams were maintained with their litters until the pups were of the desired age (7- and 14-days-old).

Rats to be studied at 42 days of age were weaned from the dam at 21 days and randomly separated into groups of 5 per cage. At the desired age, rats were decapitated, and adrenal glands were quickly removed and placed into ice cold Krebs-HEPES-calcium buffer. The adrenal capsules were then manually separated from the subcapsules and each was digested in collagenase (4 mg/ml, Worthington Biochemical Corp., Freehold, NJ, USA). This separation allowed for the study of both isolated ZG cells (capsules) and of zona fasciculata/reticularis (ZFR) cells (subcapsules). Each batch of cells was counted and assessed for viability by trypan blue exclusion, both before and after the experimental incubation period. The dispersed cells were then placed in fresh buffer containing bovine serum albumin (1 mg/ml, Sigma Chemicals, St Louis, MO, USA) at a concentration of 100 000 cells/ml. Cells were always studied the day they were dispersed. The following numbers of animals were used for each separate experiment: 40 pups at 7 days of age, 30 pups at 14 days of age, and 20 weanlings at 42 days of age.

Hypoxia *in vitro* (assessment of entire steroidogenic pathway)

Buffer (2 ml) was placed into 16 separate 10 ml glass vials: eight vials for capsular cells and eight vials for subcapsular cells. Within each of these cell-type groups, two vials each were gassed with 21, 5, 1, or 0% O_2 . Within each gas mixture group, one vial received ACTH stimulation (20 ng/ml, Peninsula Labs, Belmont, CA, USA) and one vial did not (basal release). Therefore, for each experiment performed, the following comparisons were made: capsule vs subcapsule, 21 vs 5 vs 1 vs 0% O_2 , and no ACTH vs ACTH. All incubations were performed in a shaker bath at 37 °C. Gases were allowed to equilibrate in the vials until the appropriate % O_2 was measured with an O_2 electrode (Microelectrodes, Inc., Bedford, NH, USA). Cell suspensions were then added to the appropriate vials to a concentration of 50 000 cells/ml and agitated for 1 minute. Samples were drawn directly from the vials with a syringe at different time points (0 (pre-ACTH), 10, 20, 60, and 120 min post-ACTH) and quickly centrifuged to separate cells from buffer. The % O_2 in the cell medium was monitored with the O_2 electrode. The supernatants were transferred to new tubes and frozen at -20 °C until further analysis. Additional sets of experiments were performed using 2% O_2 to complete the data set in cells from 42-day-old rats because of the interesting results obtained from initial experiments.

Assessment of isolated steroidogenic enzyme activity

The set up for these experiments was identical to that found above. Each of the 16 vials received cyanoketone (10 μ M, donated by Sterling Winthrop, Collegeville, PA, USA), a potent inhibitor of 3 β -hydroxysteroid dehydrogenase (3 β -HSD, the enzyme responsible for the

conversion of pregnenolone to progesterone). The early and late steroidogenic pathways were thus dissociated which allowed the analysis of the activity of the rate-limiting enzymes.

Early pathway: P450 scc activity The mitochondrial side-chain cleavage enzyme, present in both the ZG and the ZFR, catalyzes the conversion of cholesterol to pregnenolone. Vials from both cell types under each gas concentration were stimulated with dibutyl-cAMP (3 mM, Sigma Chemicals, St Louis, MO, USA). Enzyme activity was assessed by measuring the concentration of accumulated pregnenolone in the cell medium at various time points. This approach is also dependent on endogenous substrate (cholesterol) transport into the mitochondria.

Late pathway: P450 aldo and P450c11 β activities

The late pathway involves the conversion of progesterone to 11-deoxycorticosterone (by 21 OHase) in both zones. The next step in the rat ZG is the conversion of 11-deoxycorticosterone to aldosterone, catalyzed by P450 aldo. The conversion of progesterone to 11-deoxycorticosterone is not rate-limiting. Therefore, P450 aldo activity was assessed by adding exogenous progesterone (3.2 μ M, Sigma Chemicals, St Louis, MO, USA) and measuring the concentration of aldosterone in the cell medium over the 2 h incubation. P450c11 β catalyzes the conversion of 11-deoxycorticosterone to corticosterone in the ZFR. Since 21 OHase activity is not rate limiting, we assessed P450c11 β activity by adding progesterone as above and measuring corticosterone production.

Hormone/cAMP assays

The concentrations of aldosterone, corticosterone and pregnenolone were measured by previously described RIA protocols (Raff *et al.* 1986, Raff & Chadwick 1986). The accumulation of cAMP in the cell medium was measured by RIA (Amersham, Piscataway, NJ, USA) as described previously (Matthys *et al.* 1998).

Statistical analysis

Data were analyzed by two-way analysis of variance for repeated measures and Duncan's multiple range test (SigmaStat 2.03). $P < 0.05$ was considered significant. Data are presented as means $\pm$ S.E.M.

Results

Figure 1 shows ACTH-stimulated aldosterone production from isolated ZG cells as a function of time. Each panel corresponds to cells from a different age group. Aldosterone production from 7- and 14-day-old cells was

Figure 1 ACTH-stimulated aldosterone production from rat zona glomerulosa (ZG) cells exposed to decreasing levels of O₂ as a function of time. ACTH (20 ng/ml) was added to the cell medium at $t=0$. Vertical lines above and below data points represent S.E.M. *Value significantly different from basal (0 min) value at the corresponding %O₂ with $P < 0.05$. Four separate experiments were performed for each age group.

inhibited by decreased O₂ in a concentration-dependent manner. Five percent O₂ (~35 mmHg) resulted in a 40% decrease in aldosterone production whereas 1% O₂ (~7 mmHg) resulted in an almost complete inhibition of aldosterone production after 120 min of incubation. Surprisingly, even at 0% O₂ there was some production of aldosterone, albeit small (8% of max). Aldosterone production from cells from 42-day-old rats was less sensitive to

Figure 2 ACTH-stimulated cAMP production from rat ZG cells exposed to decreasing levels of O_2 . ACTH (20 ng/ml) was added to the cell medium at $t=0$. Vertical lines above data points represent S.E.M. *Value significantly different from basal (0 min) value at the corresponding $\%O_2$ with $P<0.05$. Four separate experiments were performed for each age group.

inhibition by decreased O_2 . Steroid production at 5% O_2 was not statistically different from 21% O_2 . Furthermore, 1% O_2 resulted in a 35% decrease in aldosterone production, which was less inhibitory than that found in cells from suckling rats. Also worth noting is the increase in maximal aldosterone production in cells from older rats, as well as the latency in the response to ACTH with advancing age.

Figure 2 depicts ACTH-stimulated cAMP production from ZG cells from 7- and 42-day-old rats as a function of time. ZG cells from 7-day-old rats showed significant inhibition of cAMP production only at O_2 levels below 5%. cAMP production from cells from 42-day-old rats showed the same characteristics as the corresponding steroid production depicted in Fig 1. At 5% O_2 cAMP production was not statistically different from that at 21% O_2 , it then significantly decreased at O_2 levels of 1 or 0%. Cells from both age groups were able to mount rapid, but transient, increases in cAMP at 0% O_2 , although only 42-day-old cells showed significant increases from $t=0$.

Figure 3 ACTH-stimulated aldosterone production (% max) from rat ZG cells as a function of $\%O_2$ (top panel). ACTH-stimulated cAMP production (% max) from ZG cells as a function of $\%O_2$ (bottom panel). Vertical lines above and below data points represent S.E.M. *Value significantly different from control value (21% O_2) for corresponding age group with $P<0.05$. +Value significantly different from value at next highest experimental $\%O_2$, for corresponding age group, with $P<0.05$. Four separate experiments were performed for each age group.

Results of all measurements were similar for cells from 7- and 14-day-old rats (data not shown).

Figure 3 summarizes ACTH-stimulated aldosterone and cAMP production as a function of $\%O_2$ (data from Figs 1 and 2). Aldosterone and cAMP values were measured at the 120 min time point of the experiment and normalized as % of maximum. Normalization of data was performed so that 2% O_2 experiments in 42-day-olds (additional studies) could be displayed on the same graph. The estimated $\%O_2$ for half-maximal aldosterone production for 7- and 42-day-old rats was approximately 4% (~ 28 mmHg) and 1% O_2 (~ 7 mmHg) respectively. Also worth noting were the significant decreases in aldosterone production observed when values between 1% and 0% O_2 (42-day-old) and between 5% and 1% O_2 (7-day-old) were compared. cAMP levels in 7-day-old rat cells

Figure 4 ACTH-stimulated corticosterone production from rat zona fasciculata/reticularis (ZFR) cells as a function of time. ACTH (20 ng/ml) was added to the cell medium at $t=0$. Vertical lines above and below data points represent S.E.M. *Value significantly different from basal value (0 min) for the corresponding %O₂ with $P<0.05$. Four separate experiments were performed for each age group.

decreased as the %O₂ was lowered from 21% to 5%, with significant decreases from control occurring at levels below 5% O₂. cAMP levels from 42-day-old cells exhibited a similar concentration–response curve to that of aldosterone production for the same age group. The %O₂ for half-maximal cAMP production from the ZG of both 7- and 42-day-old rats was estimated at around 2% O₂ (~14 mmHg). Also worth noting was the significant decrease in cAMP production between 5% and 2% O₂ (42-day-old).

Figure 4 depicts ACTH-stimulated corticosterone production from ZFR cells as a function of time. Cells from 7-day-old rats exhibited a 30% decrease in corticosterone production at 5% O₂ and a further decrease (80%) at 1% O₂ (measured at 120 min). Forty-two-day-old cells did not exhibit sensitivity to decreased O₂ until 0% O₂ was reached, at which point corticosterone production was 30% of maximum. For 7-day-old cells, significant increases from $t=0$ occurred only at 21% and 5% O₂. The data also illustrated the large increase in maximal steroidogenesis that occurs in older rats. Maximal values under 21% O₂ for 42-day-old cells were nearly 30 times higher than that for 7-day-old cells.

Figure 5 ACTH-stimulated corticosterone production (% max) from ZFR cells as a function of %O₂. Vertical lines above and below data points represent S.E.M. *Value significantly different from control value (21% O₂) for the corresponding age group with $P<0.05$. *Value significantly different from value at next highest experimental %O₂, for corresponding age group, with $P<0.05$. Four separate experiments were performed for each age group.

The concentration–response curve for %O₂ and corticosterone production is depicted in Fig. 5. Estimated %O₂ for half-maximal corticosterone production was 4% O₂ for 7-day-old cells and less than 1% O₂ for 42-day-old cells. Because of a significant change by the vendor of the cAMP assay methodology, we were unable to complete cAMP measurements in subcapsule experiments from 7-day-old rats. Data from 42-day-old cells showed no significant changes in cAMP across the levels of O₂ used (not shown).

Figure 6 depicts steroidogenic enzyme activity in the ZG. The top panel shows cAMP-stimulated pregnenolone production from endogenous precursors after 120 min incubation as a function of %O₂ (early pathway: P450 scc activity). ZG cells from 42-day-old rats displayed significant decreases (49%) in P450 scc activity (as compared with that at 21% O₂) when O₂ levels were decreased to 5% (there was no further inhibition below 5% O₂). P450 scc activity in 7-day-old ZG cells was significantly increased (81%) from control at 5% O₂, and then significantly decreased (34%) between 5% and 1% O₂. The lower panel displays the conversion of progesterone to aldosterone after 120 min incubation as a function of %O₂ (late pathway: P450 aldo activity). P450 aldo activity was essentially unaffected by decreases in O₂ from 21% to 5% (both age groups), but decreased dramatically at lower O₂ levels. The decreases in aldosterone production observed between 5% and 1% O₂ for 7- and 42-day-olds were 75% and 51% respectively. Early and late steroidogenic enzyme activities were also found to be higher in cells from older rats, as noted for maximal steroid output.

The assessment of P450 scc and P450c11 β enzyme activities in the ZFR is depicted in Fig. 7. The top panel

Figure 6 P450 scc (top panel) and P450 Aldo (bottom panel) activities in rat ZG cells exposed to decreasing levels of O₂. Vertical lines above and below data points represent S.E.M. *Value significantly different from control (21% O₂) for the corresponding age group with $P < 0.05$. Value significantly different from value at next highest experimental %O₂ for corresponding age group, with $P < 0.05$. Three separate experiments were performed for each age group. The y-axis for each graph was log transformed to accommodate the range of data gathered from each age group.

showed significant decreases (62%) in P450 scc activity in cells from 42-day-old rats at O₂ levels of 5% (as compared with 21% O₂). There was no further inhibition of P450 scc activity at levels of O₂ below 5%. P450 scc activity in 7-day-old ZFR cells showed a very similar concentration-response curve to that of P450 scc activity in the 7-day-old ZG. Activity was increased by 40% when O₂ was lowered from 21% to 5%, and then decreased significantly between levels of 5% and 1% O₂. The lower panel shows no significant change in P450c11 β activity in 42-day-old cells across the range of O₂ studied. However, 7-day-old ZFR cells displayed significant decreases (38%) in P450c11 β activity when the %O₂ was decreased from control (21%) to 5%. There were no significant decreases in P450c11 β at levels of O₂ below 5%. The lower panel also illustrates the aforementioned higher steroid production in older rats.

Figure 7 P450 scc (top panel) and P450c11 β (bottom panel) activities in rat ZFR cells exposed to decreasing levels of O₂. Vertical lines above and below data points represent S.E.M. *Value significantly different from control (21% O₂) for the corresponding age group with $P < 0.05$. Three separate experiments were performed for each age group. The y-axis for each graph was log transformed to accommodate the range of data gathered from each age group.

P450c11 β activity was nearly an order of magnitude higher in cells from 42-day-old rats when compared with that of 7-day-old rats.

Table 1 shows basal (no ACTH) aldosterone levels at the 120 min time point of the experimental incubation. Significant changes in basal aldosterone were observed in ZG cells only when 0% O₂ was reached. This was true for cells at all three ages. The table also illustrates an increase in basal aldosterone production with advancing age.

Initial cell viabilities (10⁶ cell/ml) ranged from 2.8 to 4.0, with increased cell concentration observed in ZFR preparations as well as in preparations from older rats. There was no significant decrease in cell viability following a 2 h exposure to 0% O₂ regardless of age, cell type, or treatment (data not shown).

Table 1 Basal aldosterone production (ng/10⁶ cells per h) at 120 min of incubation

	Age (days)		
	7	14	42
%O ₂			
0	1.6 ± 0.5*	2.4 ± 0.5*	22.4 ± 4.6*
1	3.1 ± 0.7	5.5 ± 1.3	52.7 ± 27.6
5	4.1 ± 1.0	7.4 ± 1.3	132.0 ± 43.9
21	5.1 ± 1.1	6.6 ± 1.0	88.4 ± 31.5

Basal aldosterone production (no ACTH) from zona glomerulosa (ZG) cells following 120 min of incubation at 37 °C as a function of O₂. Results are given as the mean ± S.E.M. *Value significantly decreased from control (21% O₂) for that corresponding age group. Four separate experiments were performed for each age group.

Discussion

The purpose of this study was to determine the effects of decreasing O₂ concentrations on steroidogenesis from rat adrenocortical cells *in vitro*. Previous studies examined the effects of hypoxia on steroidogenesis in a variety of species (Raff 1991, Brickner *et al.* 1992, Raff *et al.* 1996, Raff *et al.* 1999). We chose to study adrenal cells from rats at two distinct stages of development. Most previous studies have examined the effects of hypoxia on steroidogenesis *in vivo* (Raff & Chadwick 1986, Raff *et al.* 1986, Raff *et al.* 1999). We wanted to examine the effects of acute hypoxia *in vitro* and compare them with results previously found for bovine cells (Raff & Kohandarvish 1990, Brickner *et al.* 1992). The cellular mechanisms involved in ACTH-stimulated steroidogenesis were given close attention.

Acute decreases in O₂ and the zona glomerulosa

These studies found ACTH-stimulated steroid production from rat adrenocortical cells to be decreased when cells were subjected to decreased O₂ concentrations. This effect was most pronounced in the ZG of both 7- and 42-day-old rats, a zonal specificity that has been shown experimentally to exist in both adult humans and adult rodents (Slater *et al.* 1969, Raff 1991). Aldosterone production was greatly inhibited in cells from 7-day-old rats as compared with those of 42-day-olds.

These initial findings clearly indicated that the rat ZG, regardless of age, is much more resistant to hypoxia than the bovine ZG. Estimated P₅₀ values for aldosterone production were 28 mmHg for 7-day-old and 7 mmHg for 42-day-old rat cells. These values are quite remarkable when compared with the P₅₀ obtained for bovine cells, estimated to be around 95 mmHg (~13% O₂) (Raff & Kohandarvish 1990). This difference is not surprising considering the significant difference between these two species in the control of steroidogenesis and enzyme expression (Muller 1991).

cAMP has long been known to be the main second messenger involved in ACTH-stimulated aldosterone production (Haynes 1958, Cote *et al.* 1999). The accumulation of cAMP in the cell medium was measured following ACTH-stimulation under varying levels of O₂, and these values were used as an index of cAMP generation (Matthys *et al.* 1998). ACTH-stimulated aldosterone production from 7-day-old ZG cells was significantly decreased at levels of O₂ below 21%. This could be partially explained by changes in cAMP generation. ACTH-stimulated cAMP production from 7-day-old cells was not significantly decreased (from control) until cells were exposed to 1% O₂. Significant decreases in aldosterone production from 42-day-old cells did not occur until the concentration of O₂ was lowered to 1%. cAMP production from 42-day-old ZG cells was significantly decreased at 2% O₂. Therefore, decreases in cAMP could be at least partially responsible for the observed decreases in aldosterone production.

It was of interest to determine if the early pathway (including cholesterol transport) was affected by hypoxia, independent of any observed changes in cAMP (Papadopoulos *et al.* 1997, Stocco & Clark 1997, Zilz *et al.* 1999). It would seem that the decreases in aldosterone production observed between 5% and 1% O₂ (7-day-old ZG) could be explained by decreased P450 scc activity, decreased cholesterol delivery, or a combination of both. Previous research lends support to the idea that the O₂-dependence of P450 scc is actually due to a reduction in cholesterol delivery under hypoxic conditions (Stevens *et al.* 1984). Future experiments using permeable forms of cholesterol as a substrate will provide answers regarding isolated activity of P450 scc.

Previous bovine studies performed *in vitro* found that low O₂ specifically inhibited the late pathway P450 aldo (Brickner *et al.* 1992). Other studies using bovine cells found that the activity of another cytochrome enzyme, P450c17 α , was also sensitive to decreases in O₂ (Chabre *et al.* 1993). Our results showed significant decreases in P450 aldo activity at O₂ levels below 5% (both age groups). Therefore, in ZG cells from 42-day-old rats, decreased aldosterone production under acute hypoxia could be attributed to decreased P450 aldo activity, as shown for bovine cells. Decreases in aldosterone production from 7-day-old ZG cells under low O₂ could be explained, at least partially, by decreased P450 aldo activity.

Our results indicate that aldosterone production from 42-day-old ZG cells is inhibited only by severe decreases in O₂. This inhibition seems to take place at the level of P450 aldo, and the decrease in P450 aldo activity could be due to the direct inhibition of P450 aldo. Reduced substrate availability could be due to decreases in molecular O₂ or a reduction in equivalent generation via the respiratory chain (NADH). However, these mechanisms of inhibition may not be the only explanations for the

observed decreases in P450 aldo activity. An alternative explanation could be that P450 aldo activity was inhibited by increased release of nitric oxide (NO) (Hanke & Campbell 2000).

What mechanisms could explain the decrease in aldosterone production observed in 7-day-old cells as O₂ was lowered from 21% to 5%? Although the results seem to indicate that decreased cAMP production was responsible for this decrease, it has been shown that ACTH-stimulated steroid production in the rat ZG may depend on non-cAMP mechanisms (Gallo-Payet *et al.* 1999). These include such mechanisms as the phospholipase C/protein kinase C pathway and related intracellular fluxes in Ca²⁺ concentration (Durrux *et al.* 1991, Gallo-Payet *et al.* 1996, Gallo-Payet *et al.* 1999). It is possible that hypoxia alters the response of these separate signaling pathways to ACTH. Likewise, changes in mitochondrial function and/or properties may also play a role in decreased ACTH-stimulated aldosterone production during acute hypoxia. Previous studies utilizing rabbit kidney tubule cells found that hypoxia caused a change in mitochondrial electrochemical gradient (designated Ψ_m) induced by decreases in aerobic respiration (Weinberg *et al.* 2000). These findings would lend support to other studies that found that the delivery of cholesterol (via steroidogenic acute regulatory protein (StAR)) to the inner mitochondrial membrane was dependent on an intact Ψ_m (King *et al.* 1999).

Acute decreases in O₂ and the zona fasciculata/reticularis

ACTH-stimulated corticosterone production from 7-day-old ZFR cells was affected by decreased O₂ in a concentration-dependent manner. The P₅₀ for ACTH-stimulated corticosterone production from 7-day-old cells was approximately 28 mmHg. The corticosterone response of 7-day-old ZFR cells to ACTH stimulation under decreasing levels of O₂ was quite similar to the aldosterone response of 7-day-old ZG cells. Forty-two-day old ZFR cells were by far the most resistant to inhibition of ACTH-stimulated steroidogenesis by decreased O₂. The P₅₀ for corticosterone production in 42-day-old ZFR cells was actually less than 7 mmHg. When ACTH-stimulated cAMP production from 42-day-old ZFR cells was assessed, results indicated no significant changes across the range of O₂ studied.

The concentration–response curves for P450 scc activity (early pathway) and %O₂ in ZFR cells were similar to the curves found for ZG cells (both age groups). Given these findings, it is likely that changes in ZFR P450 scc activity due to hypoxia are at least partly responsible for the decrease in corticosterone production observed between 5% and 1% O₂ (7-day-old cells). The activity of P450c11 β in ZFR cells was only slightly affected by decreases in O₂. P450c11 β activity in 7-day-old cells was significantly decreased (38%) when the %O₂ was lowered from 21% to

5%. However, further decreases in O₂ to levels approaching 0% had no significant effect on P450c11 β activity in these neonatal cells.

The effect of acute hypoxia on ZFR cells shows similarities to its effect on aldosterone production from ZG cells, but the mechanisms differ slightly. The effects of decreased O₂ on 7-day-old ZFR corticosterone production could be partially explained by decreases in P450c11 β activity (between 21% and 5% O₂) and decreases in P450 scc activity (between 5% and 1% O₂). But, as with findings for ZG cells, decreases in enzyme activity must be explained further (e.g. NO and mitochondrial alterations). A study examining the effects of NO on rat ZFR cells found that increases in intracellular NO production specifically inhibited corticosterone production by decreasing the activity of P450 scc (Cymeryng *et al.* 1998). However, as stated earlier, a decrease in P450 scc activity may not necessarily be due to a direct effect of low O₂ on the enzyme itself.

One possible explanation could be an alteration in StAR activity under hypoxia. Previous research performed *in vivo* has shown that acute increases in corticosterone production (1–2 h post-ACTH stimulation) may be due to small, but significant, increases in StAR activity (Lehoux *et al.* 1999). The findings indicated the presence of multiple isoforms of the StAR protein, each having a unique isoelectric point (pI). It could be possible that some cellular effect of hypoxia, perhaps small changes in pH, overrides the stimulatory action of ACTH on StAR-mediated cholesterol transport. This inhibition could depend on the StAR isoforms present in the cell, and could be hypothesized to occur in ZG cells as well as in ZFR cells.

The observed decreases in corticosterone production from 7-day-old cells could also be explained by changes in Ca²⁺ dynamics, as mentioned earlier with regards to the ZG. Previous research has shown that calcium channel blocking agents, such as verapamil, significantly decreased both ACTH and cAMP-stimulated corticosterone production (Matsuki *et al.* 1996). The stimulatory and permissive actions of intracellular Ca²⁺ have been very well established (Mahaffee & Ontjes 1980, Cheitlin *et al.* 1985, Ganguly & Davis 1994, Gallo-Payet *et al.* 1996) and inhibitory properties of Ca²⁺ have been proposed as well (Cooper *et al.* 1995). Most of these studies found a strong interaction between Ca²⁺ and cAMP.

Decreases in corticosterone production in ZFR cells from 42-day-old rats did not occur until the level of O₂ approached 0%. This occurred without significant changes in enzyme activities or cAMP dynamics. What could be a possible explanation for this decrease in corticosterone production? Perhaps at such low O₂ tension the cells simply do not survive or have severely diminished function. The fact that cell viability was not significantly altered after 2 h under 0% O₂ argues against the former. Although there was no significant decrease in P450c11 β

activity to explain the decreased corticosterone production (at 0% O₂), the fact that its activity was studied in the presence of excess substrate (progesterone) could explain the discrepancy. Previous research studying the effects of hypoxia on placental villi found that the activity of 3 β -HSD was reduced under low O₂ (Gabbe & Vilee 1971). Therefore, the decreased corticosterone production from 42-day-old ZFR cells at 0% O₂ may be due to 3 β -HSD inhibition by low O₂.

How does the stress hyporesponsive period (SHP) relate to the present study? Adrenocortical cells from 7-day-old rats, when exposed to decreasing O₂, showed similar dose-dependent decreases in ACTH-stimulated aldosterone and corticosterone production. Since the same concentration of ACTH was used across all levels of O₂ studied, it provides evidence that hypoxia further decreases responsiveness to ACTH through the aforementioned mechanisms. Decreased P450 scc activity in neonatal cells at low levels of O₂ (<5%) may be augmented by the decreases in peripheral-type benzodiazepine receptor (PBR) expression (reduced cholesterol transport) normally found at this age (Zilz *et al.* 1999). It is also possible that hypoxia indirectly affects PBR activity, independently of gene expression (e.g. Ca²⁺ flux).

Theoretical interpretation of the results

The concentration of O₂ present in the atmosphere (21% O₂; ~150 mmHg) was used as a control. Other *in vitro* studies have also used this O₂ concentration as a control (Raff *et al.* 1989, Brickner *et al.* 1992, Raff & Jankowski 1995). However, the PO₂ of arterial blood is ~95 mmHg (Raff *et al.* 1986). Studies performed in normoxic dogs determined the PO₂ of adrenal venous blood to be ~70 mmHg (Breslow *et al.* 1990). Since venous PO₂ reflects capillary PO₂, it may be more relevant to consider a concentration of about 10% O₂ (~70 mmHg) as a control value when studying cells *in vitro*. The large gap in O₂ concentrations used in the current experiments (between 5% and 21% O₂) preclude any comparisons using 10% O₂ as a control.

There is always controversy concerning whether PO₂ values used in *in vitro* studies represent physiologically relevant O₂ levels (Jones & Kennedy 1982). Although adrenal venous PO₂ in the normoxic animal is probably higher than the mixed venous PO₂, it is not clear what it is in hypoxic animals when arterial PO₂ is <40 mmHg. Adrenal venous PO₂ was measured, but not explicitly reported, in a previous study of hypoxic dogs (Breslow *et al.* 1989); we were nevertheless able to estimate that it was <10–20 mmHg based on reported adrenal blood flow and oxygen extraction. Therefore, the oxygen levels in the present study may be quite representative of venous and hence capillary PO₂ in the adrenal gland *in vivo* during hypoxia.

In addition, the PO₂ at the mitochondria is unknown for adrenocortical cells. However, for other tissues, it may be as low as 1 mmHg or even lower (Jones & Kennedy 1982). Therefore, it may be that the P₅₀ for steroidogenesis in bovine ZG cells *in vitro* is unusually high and that a P₅₀ <10 mmHg in the rat, as we have reported here, is a better representation of mitochondrial PO₂ in other mammalian adrenal glands. Regardless, it is clear that there is a developmental change in the sensitivity of adrenocortical cells to hypoxia with age that is worth pursuing.

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