Steroidogenesis in human aldosterone-secreting adenomas and adrenal hyperplasias: effects of hypoxia in vitro

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**Raff, Hershel, Eric D. Bruder, and the St. Luke's Medical Center Adrenal Tumor Study Group.** Steroidogenesis in human aldosterone-secreting adenomas and adrenal hyperplasias: effects of hypoxia in vitro. *Am J Physiol Endocrinol Metab* 290: E199–E203, 2006. First published August 16, 2005; doi:10.1152/ajpendo.00337.2005.—The synthesis of adrenal steroids requires molecular oxygen. Because arterial hypoxemia is a common clinical condition, the purpose of the present study was to examine steroidogenesis in vitro under physiological changes in O2 tension (PO2) in cells from human adrenal glands with aldosterone-secreting adenomas (ASA; n = 3) or with bilateral adrenal hyperplasia causing Cushing’s syndrome (n = 4). A decrease in PO2 from 150 mmHg (mild hypoxia) to 80 mmHg had minimal effect on steroid production. A reduction to 40 mmHg (still well within the physiological range) significantly inhibited cAMP- and ACTH-stimulated aldosterone, cortisol, and dehydroepiandrosterone (DHEA) production from ASA. Furthermore, cortisol and DHEA production in cells from histologically normal tissue, adjacent to ASA and from bilateral adrenal hyperplasias, was also inhibited under a PO2 of 40 mmHg. We conclude that physiological decreases in PO2 to levels typical for adrenal venous PO2 under mild hypoxia inhibit steroidogenesis. These studies may have implications for oxygen therapy in critically ill patients with functional adrenal insufficiency, as well as for therapeutic options in patients with adrenal neoplasms.

**METHODS**

**Tissue source and isolation of cells.** This study was approved by the Institutional Review Board of Aurora Health Care, and all patients gave signed informed consent. Adrenal glands were obtained during clinically indicated laparoscopic unilateral or bilateral adrenalectomy. Tissue was obtained from patients with either clinically established and biochemically verified primary aldosteronism due to ASA (n = 3) or Cushing’s syndrome due to bilateral adrenal hyperplasia (BAH; n = 4). Resected tissue was immediately examined by the pathologist. Adrenals from patients with ASA were sectioned and then designated as grossly normal adjacent adrenocortical or nodular tissue. Patients with BAH were identified preoperatively, and the diagnosis was confirmed by the pathologist in the operating room (in all cases, final histopathology confirmed the tissue assignments). After sectioning, tissue was placed in ice-cold Krebs-HEPES-calcium buffer containing bovine serum albumin (1 mg/ml). Tissue samples were then digested with collagenase (4 mg/ml) in fresh BSA buffer (37°C). Cells were harvested every 30 min during a 90-min incubation. Each batch of cells was counted and assessed for viability by trypan blue exclusion, counting only cells with visible lipid droplets. The dispersed cells were then placed in fresh buffer and diluted to an experimental concentration of 100,000 cells/ml.

**Assessment of steroidogenesis in vitro.** An aliquot of newly dispersed cells was placed in 5-ml polystyrene test tubes. Experimental treatments included the following: control, ACTH (0.2 or 2.0 ng/ml), or dibutyryl cAMP (1.0 mM). All treatments were performed in triplicate. Cells were incubated for 2 h under metal tents in a shaker-water bath at 37°C. These tents were vented with room air (21% O2) or low O2 (10% or 0%) in N2. Because the metal tents were much more sensitive to decreases in oxygen compared with cortisol production (18). We had proposed that a decrease in aldosterone during hypoxia would have beneficial diuretic effects, although the maintenance of cortisol production would be necessary to sustain metabolic and vascular function (16, 17, 19). Others have also demonstrated O2-sensitive steroidogenesis in a variety of in vitro steroidogenic cell models (4, 6, 9, 23).

Finally, the role of hypoxia in solid tumors and the interaction of oxygen delivery and chemotherapy have been extensively evaluated (24, 26). It has been suggested that manipulation of oxygen sensitivity with chemical agents may play a role in the treatment of solid tumors (21, 26, 27). Therefore, this initial study has evaluated the sensitivity of dispersed cells from adenomas and histologically normal adjacent tissue, as well as from bilateral adrenal hyperplasia, to a decrease in PO2 within the physiological range as well as to mild hyperoxia.

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a flow-through system, room air was not completely excluded. Therefore, the resultant O₂ tension (P₀₂) levels were 150 mmHg for 21% O₂, 80 mmHg for 10% O₂, and 40 mmHg for 0% O₂ measured by oxygen electrode (Radiometer ABL2). The experiment was stopped by placing the tubes on ice and then centrifuging the cells at 4°C. Supernatants were frozen for further analysis.

**Measurement of steroids.** Cortisol was measured by radioimmunoassay (RIA; Diagnostic Products, Los Angeles, CA) as used previously (18). DHEA was measured by enzyme immunoassay (Diagnostic Systems Laboratories, Webster, TX). Aldosterone was measured by RIA as used previously (3). The accumulation of steroid in the cell medium (final concentration) was used as an assessment of steroid synthesis and release from the cell.

**Chemical reagents.** HEPES, BSA, and cAMP were purchased from Sigma Chemical (St. Louis, MO). Collagenase was purchased from Worthington Biochemical (Freehold, NJ). ACTH-(1–39) was purchased from Peninsula Laboratories (Belmont, CA). All other chemicals were of reagent grade and were purchased from Sigma or Fisher Scientific (Fair Lawn, NJ).

**Statistical analysis.** All data were analyzed by two-way ANOVA for repeated measures and Duncan’s multiple range test for multiple comparisons (SigmaStat 2.03). P < 0.05 was considered significant. Data are presented as means ± SE. Basal and peak steroid production from adenomas and adjacent tissue varied quite widely between experiments. Therefore, these results are expressed as a percentage of basal steroid production at 21% O₂. Basal steroid production was compared between different cell types by ANOVA on ranks (SigmaStat 2.03). Data from BAH tissue were analyzed after logarithmic transformation and are presented on a logarithmic scale.

### RESULTS

Table 1 shows basal (control) steroid concentrations in the supernatant of incubated cells at 21% O₂ (P₀₂ = 150 mmHg). Aldosterone levels were undetectable in supernatants from normal tissue adjacent to ASAs and in three of four cell preparations from BAH. There were no significant differences in basal steroid concentrations between cell types. The high variability between cell preparations led us to normalize the data from ASA and adjacent tissue to the control steroid concentrations shown in Table 1.

Figure 1 depicts aldosterone production by cells from aldosterone-producing adenomas studied in vitro. Basal aldosterone production was not inhibited by a decrease in P₀₂ to 80 or 40 mmHg. cAMP (1.0 mM) and both concentrations of ACTH significantly increased aldosterone production in the control (150 mmHg) oxygen exposure. ACTH and cAMP-stimulated aldosterone production was inhibited by a decrease in P₀₂ to 40 mmHg. There were very low or undetectable levels of aldosterone production from cells obtained from tissue adjacent to the adenoma.

Figure 2 depicts cortisol production by cells from ASA (Fig. 2A) and histologically normal tissue adjacent to these adenomas (Fig. 2B). ACTH (2.0 ng/ml) and cAMP both elicited significant increases in cortisol production at all levels of oxygen studied. The lower concentration of ACTH did not result in a significant increase in cortisol except under a P₀₂ of 150 mmHg. The inhibition of the cortisol response to either cAMP or ACTH (2.0 ng/ml) was related to the decrease in oxygen. Comparison of cortisol production in cells from adjacent tissue (Fig. 2B) with cells from aldosterone-producing adenoma (Fig. 2A) demonstrated that the magnitude of the cortisol response to cAMP was greater than the cortisol response to ACTH in adenomas but not in adjacent tissue. However, cells from both adjacent tissue and adenomas were relatively resistant to a decrease in P₀₂ to 80 mmHg, whereas adenomas showed a much larger decrease in cAMP-stimulated cortisol production under a P₀₂ of 40 mmHg.

Figure 3 depicts DHEA production from cells from ASA (Fig. 3A) and adjacent tissue (Fig. 3B). cAMP significantly increased DHEA production from ASA at all levels of oxygen (Fig. 3A). Only the lowest P₀₂ (40 mmHg) led to an inhibition of DHEA production from cells from ASA. Cells from adjacent tissue (Fig. 3B) displayed similar patterns in DHEA production (under each treatment) compared with cortisol (Fig. 2).

**Table 1. Basal steroid concentrations (ng/ml) in supernatants from dispersed human adrenal cells (150 mmHg)**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Aldosterone-Secreting Adenomas (n = 3)</th>
<th>BAH (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenoma</td>
<td>Adjacent Tissue</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>7.4 ± 0.7</td>
<td>ND</td>
</tr>
<tr>
<td>Cortisol</td>
<td>18 ± 10</td>
<td>49 ± 20</td>
</tr>
<tr>
<td>DHEA</td>
<td>0.4 ± 0.2</td>
<td>8.5 ± 6.2</td>
</tr>
</tbody>
</table>

All steroid concentrations are means ± SE in ng/ml. BAH, bilateral adrenal hyperplasia; DHEA, dehydroepiandrosterone; ND, aldosterone levels were very low or undetectable. Otherwise, there were no statistically significant differences within each steroid concentration between tissue types.

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Fig. 1. Aldosterone responses as %control (no secretagogue; 150 mmHg) to cAMP or ACTH in cells from aldosterone-secreting adenomas (n = 3). Cells were incubated under a P₀₂ of 150 mmHg, 80 mmHg, or 40 mmHg. *Significant increase from control under the same P₀₂; **significant inhibition compared with the same secretagogue concentration under 150 mmHg.
cAMP and ACTH (2.0 ng/ml) under a PO2 of 40 mmHg; however, these increases were attenuated compared with incubation under a PO2 of 150 mmHg. Changes in DHEA production (Fig. 4B) in response to stimuli or oxygen level nearly mimicked those found for cortisol production. Within each treatment, decreasing PO2 to 40 mmHg significantly reduced DHEA production. DHEA production in response to ACTH was not significantly increased at a PO2 of 40 mmHg compared with basal levels at the same PO2.

**DISCUSSION**

This study evaluated steroidogenesis in cells from human adrenal glands under a physiological range of PO2. Decreases in PO2 to 40 mmHg resulted in a decrease in the production of all steroids measured in cells from aldosterone-secreting adenomas and histologically normal tissue adjacent to aldosterone-secreting adenomas and bilateral adrenal hyperplasias. Hypoxia (150 mmHg) did not consistently increase steroid production compared with 80 mmHg. Of note was that aldosterone-secreting adenomas released large amounts of cortisol and DHEA in addition to aldosterone and that all steroids produced from adenomas were dramatically stimulated by cAMP. This cAMP-stimulated steroid production was partially inhibited when cells were incubated under a PO2 of 40 mmHg. Also of note, but not unexpected, was that histologically normal tissue adjacent to aldosterone-secreting adenomas did not release measurable levels of aldosterone, probably because of long-term suppression of renin, commonly found in patients with primary hyperaldosteronism (12).

First, a comment about the levels of PO2 chosen for these experiments. We were only interested in changes in PO2 within the pathophysiological range as well as under slightly hyperoxic conditions. The adrenal gland has very high blood flow per gram of tissue (1, 2). Even though it uses molecular oxygen for steroidogenesis, as well as for the maintenance of cellular function, like most endocrine organs, adrenal blood flow is considerably higher than required to maintain oxygen consumption. This is obviously to maximize hormone delivery to the systemic circulation. Measurement of adrenal venous PO2...
levels ranging from 70 mmHg under normoxic conditions to 20 mmHg under severe, acute hypoxia (1, 2). This suggests that the range of adrenal Po2 in our study is right at critical patho-physiological levels of oxygen, from mildly hyperoxic to roughly normoxic (80 mmHg) to a level that one would expect with moderate, clinical hypoxia (40 mmHg).

We have previously demonstrated that bovine, rabbit, and rat adrenocortical cells are sensitive to changes in PO2 within the physiological range (3, 15, 18). This is the first study, to our knowledge, to evaluate this phenomenon in cells from human adrenal tissue. A major difference from these previous studies to the human cells in the present study was the high sensitivity of cortisol production to modest decreases in oxygen, because our previous study in bovine cells showed that aldosterone production was more sensitive to inhibition under low oxygen compared with cortisol (18).

It is also interesting to point out that aldosterone-secreting adenomas produced large amounts of cortisol and DHEA in the basal state and dramatically increased production of these steroids with stimulation with cAMP. Although patients with aldosterone-secreting adenomas do not usually have clinical hypercortisolism or hyperandrogenism (14), this in vitro phenomenon has been described previously (13, 25) and has the potential to be helpful diagnostically (8).

**Physiological implications.** We were unable to obtain tissue from truly normal adrenal glands because they are no longer routinely removed during unilateral “nephron-sparing” nephrectomy (22). Therefore, we must extrapolate from histologically normal tissue adjacent to adrenal adenomas. In all cases, steroidogenesis was very sensitive to modest decreases in Po2, whereas hyperoxia tended to, but did not consistently, increase steroid production. We previously hypothesized that decreases in aldosterone during hypoxia might allow a beneficial natriuresis and diuresis to prevent edema (16, 17, 19). It is remarkable, therefore, that cortisol and DHEA production were also inhibited by a decrease in Po2 to 40 mmHg, and we do not yet have a teleological explanation for this. Whether this would occur during hypoxia in vivo in patients with primary increases in adrenal steroidogenesis is not known.

**Translational physiology.** One aspect of this study relevant to clinical medicine is critical illness. It is well known that critically ill patients (e.g., with sepsis) studied in an intensive care unit can have diminished adrenal function (20). The diminished adrenal function in septic, critically ill patients is more evident when they are stimulated with physiological doses of ACTH (11). One potential explanation for this that is relevant to the present study is the possibility that these critically ill patients have diminished oxygen delivery to the adrenal glands. Therapy focused on improving oxygen delivery could therefore potentially improve steroidogenesis in critically ill patients.

The sensitivity of steroidogenesis in human adrenals to modest decreases in oxygen may have implications in potential treatment options, particularly since it appears that the relatively hyperactive adenomas were inhibited by a Po2 of 40 mmHg. Manipulation of oxygen delivery to solid tumors as an adjunct to chemotherapy has been of interest for many years (21, 26, 27). In fact, there are now adjuvant therapies that are targeted to alter the sensitivity to oxygen delivery. The high endogenous sensitivity to hypoxia that we have demonstrated may suggest that adrenal tumors might be amenable to this chemotherapeutic approach. Although we did not study adrenal carcinoma, this difficult-to-treat, and often fatal, cancer may also be an appropriate target for manipulation of oxygen sensitivity during chemotherapy.

In conclusion, steroidogenesis in aldosterone-secreting adenomas, bilateral adrenal hyperplasias, and normal adrenocortical tissues appears to be sensitive to modest decreases in Po2. It remains to be seen whether this can be exploited in the therapy of adrenal neoplasms.

**ACKNOWLEDGMENTS**

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