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OXYGEN DEPENDENCE OF OESTROGEN PRODUCTION BY HUMAN PLACENTAL MICROSOMES AND CULTURED CHORIOCARCINOMA CELLS

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Summary—The oxygen dependence of oestrogen (oestrone and 17β-oestradiol) formation from androstenedione and testosterone was studied in term human placental microsomes and in cultured human choriocarcinoma cells (BeWo line). Incubations were performed under various steady-state oxygen concentrations and the production of oestrone and 17β-oestradiol quantitated by specific radioimmunoassays. The aromatization of C_{19}-steriods by both placental microsomes and choriocarcinoma cells was shown to be oxygen dependent over a wide range of O_2 concentrations. The results indicate that placental oxygenation may be a critical factor in determining oestrogen production *in vivo*. Therefore, impaired oestrogen biosynthesis due to hypoxia could be an important factor in a variety of physiological and pathological conditions.

INTRODUCTION

The production of adequate steroid hormones by the placenta is critical to the maintenance of a normal pregnancy [for reviews see references 1–2]. The biosynthesis of C_{18}-oestrogens from C_{19}-androgens is catalysed by the aromatase complex in the endoplasmic reticulum of the placenta [3–4]. This conversion involves three hydroxylation reactions and utilizes 3 moles each of NADPH and molecular O_2 per mole of oestrogen produced [5–6]. Since O_2 is required for the biosynthesis of oestrogens, a decreased supply of O_2 to the placenta due to hypoxia would be expected to have a direct effect on the production of steroid hormones. Consequently, the function of the placenta could be impaired.

Previous studies by Zachariah and Juchau[7] indicate that the synthesis of oestrone from androstenedione by human placental microsomes is very sensitive to oxygen concentrations. They found that the aromatase activity at 20% O_2 was approximately half that at 100% O_2, suggesting that the aromatase complex has a very high apparent K_m for oxygen. If such a high apparent K_m occurs *in vivo*, placental oestrogen synthesis would be critically dependent upon oxygenation since the normal arterial O_2 concentration is about 110–140 μM (75–100 torr). Thus, conditions which lower placental oxygenation such as anemia, exposure to reduced barometric pressure (high altitudes) and decreased functional hemoglobin (e.g. bound carbon monoxide), may result in altered oestrogen production.

To examine the effect of hypoxia on oestrogen production, we investigated the oxygen dependence of aromatase activity in isolated term human placental microsomes and in cultured choriocarcinoma cells under various steady-state O_2 concentrations. Two physiological substrates, androstenedione and testosterone, were studied to ascertain whether oestrogen production from different precursors was differentially affected by hypoxia. The results show a marked dependence of aromatase activity on oxygen concentration in both isolated microsomes and whole cells.

EXPERIMENTAL

Water was glass distilled. All solvents were analytical reagent grade and were used without further purification. [2,4,6,7-^{3}H] Oestrone (95.6 Ci/mmol) and [2,4,6,7,16,17-^{3}H] 17β-oestradiol (137.1 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. and were checked for purity before use by thin-layer chromatography. Fetal bovine serum (FBS), trypsin and Ham's F10 nutrient medium were obtained from GIBCO, Grand Island, NY, U.S.A. Plastic culture flasks were from Falcon Plastics, Cockeysville, MD, U.S.A. Unlabelled steriods and miscellaneous chemicals were from Sigma Chemical Co., St Louis, MO, U.S.A. Human choriocarcinoma cells (BeWo line: ATCC NO. CCL98) were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Rabbit antisera to the 3-carboxymethoxime derivatives of oestrone and 17β-oestradiol were the gifts of Dr Delwood Collins, Grady Memorial Hospital, Atlanta, GA, U.S.A. Full term human placenta were obtained from the obstetrics service of Grady Memorial Hospital, Atlanta.

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Preparation of placental microsomes

Placentas were obtained immediately after delivery and placed on ice. All subsequent operations were performed at 0–4°C. The placentas were washed with 0.01 M potassium phosphate buffer, pH 7.4, the soft tissues excised, cut into small pieces, washed again with phosphate buffer and homogenized for 3 min at full speed in a Waring blender in 5 vol of buffer. The homogenate was centrifuged at 4°C for 20 min at 10,000 *g* to remove cell debris, nuclei and mitochondria. The supernatant was centrifuged at 100,000 *g* for 1 h to obtain the microsomal pellet. The pellet was resuspended in about 40 ml of 0.01 M phosphate buffer, pH 7.4 (equivalent to 10–15 mg protein/ml), and homogenized in a Potter–Elvejhem homogenizer to give a homogeneous suspension. Two ml aliquots of the microsomal suspension were rapidly frozen in a dry ice–acetone mixture and stored at −80°C until used. Aromatase activity was stable for at least 6 months. Microsomes were prepared from BeWo cells by sonicating cell suspensions in a bath sonicator at 0°C. This was done for successive 4 ml aliquots for 15 s and repeated 3 times for each aliquot. Cells were then centrifuged at 9000 *g* for 15 min and microsomes recovered by centrifugation at 100,000 *g* for 1 h.

Culture and preparation of BeWo cells

BeWo cells were maintained as previously described [8] in Ham's F10 nutrient medium supplemented with 15% FBS (v/v) in a 95% air–5% CO_2 gas exchange incubator at 37°C. At confluency, the culture medium was discarded and the cell layers washed with 10 ml phosphate buffered saline (PBS: 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) to remove traces of FBS. A 0.25% trypsin solution (in PBS) was added to the flasks and incubated at 37°C for 20 min to obtain a uniform cell suspension. Cell suspensions were then diluted in Ca^{2+}-free Kreb's–Henseleit buffer containing 12.5 mm Hepes, 1 mM EDTA and 0.1% (w/v) bovine serum albumin, pH 7.4. Cell clumps were dispersed by gently pipetting the suspension up and down several times with a 10 ml glass pipette. Generally, cells from 5 or 6 75 cm^2 flasks were combined to give a cell density of 5×10^5 ml. Viability was ascertained by exclusion of 0.2% (w/v in Kreb's–Henseleit buffer) trypan blue. Routinely 99–100% of the cells exluded the dye.

Incubation procedure

Incubations (5×10^5 cells/ml or 0.2 mg microsomal protein/ml) were performed in Ca^{2+}-free Kreb's–Henseleit buffer containing 12.5 mM Hepes and 1 mM EDTA, pH 7.4, in rotating round-bottom flask at 37°C. The incubation buffer for the microsomal system was routinely supplemented with 0.7 mM NADPH except where indicated. Reactions were initiated by addition of 0.37 μM testosterone or androstenedione and allowed to proceed for 10 min. The oxygen dependence of oestrogen production was determined under various steady-state O_2 concentrations as previously described for isolated liver cells [9]. ATP concentrations were measured by HPLC [10] in extracts from BeWo cells incubated under identical conditions to those used for measurement of oestrogen synthesis.

Radioimmunoassays for oestrone and 17β-oestradiol

Concentrations of oestrone and 17β-oestradiol were determined using specific radioimmunoassays as previously described [11]. Reactions were stopped by addition of 5 vol of ether–ethyl acetate (2:1, v/v) and extraction of steroids into the organic phase. A 10 ml aliquot of the organic phase was evaporated to dryness under oxygen-free nitrogen and the steroids redissolved in 1 ml of 0.1 M phosphate buffer, pH 6.8, containing (on a w/v basis) 0.9% NaCl, 0.1% gelatin and 0.1% KN_3. The recoveries of oestrone and 17β-oestradiol, determined by extraction and preparation of known quantities of ^{3}H-labelled steroids as described above were 95.7 ± 3% and 98.4 ± 1% respectively ($n = 3$). A 10-point standard curve from 0–200 pg, with a sensitivity of 5 pg, was used for both oestrone and 17β-oestradiol and results were analysed by a logit/log transformation on a Northstar Model 3820 Data System computer.

RESULTS

Placental microsome studies

Figure 1 shows the time course of production of oestrone, 17β-oestradiol and total oestrogens (oestrone + 17β-oestradiol) using testosterone (0.37 μM) as substrate. While the production of oestrone was approximately linear for a period of 20 min, 17β-oestradiol production was linear for about 15 min. Similar results were obtained using androstenedione as substrate (results not shown). Regardless of the C_{19}-precursor, 17β-oestradiol production was at least 3-times that of oestrone. Consequently, *total* oestrogen production was linear for only about 15 min.

The aromatase complex of isolated microsomes exhibited a marked dependence on oxygen concentration. Similar dependences on oxygen concentrations were obtained using either androstenedione (Fig. 2) or testosterone (Fig. 3) as substrate. Half-maximal rates for total oestrogen production were obtained at approx 14 μM O_2. 17β-Oestradiol was the major product of the aromatase from both substrates at all oxygen concentration except 1 mM. The ratio of 17β-oestradiol to oestrone produced was approx 3 up to an oxygen concentration of 200 μM. At higher concentrations, the rate of production of 17β-oestradiol decreased such that at 1 mM O_2, the ratio of 17β-oestradiol to oestrone was about 1. It is important to note that *total* oestrogen production was maximal at approx 200 μM O_2.

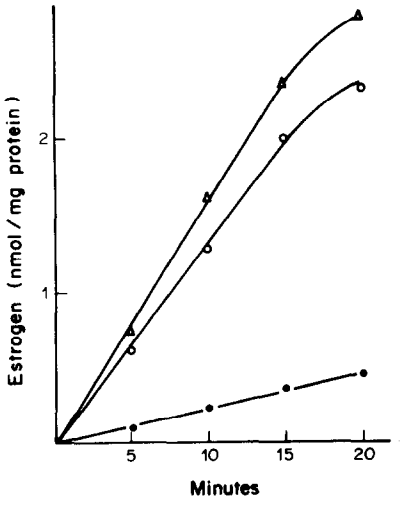

Fig. 1. Time course of oestrogen production by placental microsomes. Incubations (0.2 mg protein/ml) were performed in Ca^+-free Kreb's–Henseleit buffer containing 12.5 mM Hepes, 1 mM EDTA and 0.7 mM NADPH, pH 7.4, in rotating round-bottom flasks at 37°C. After allowing temperature equilibration for 5 min, reactions were initiated by addition of 0.37 μM testosterone. Reactions were allowed to proceed in air for the times specified and were stopped by extraction of steroids with 5 vol of ether–ethyl acetate (2:1, v/v). Oestrone and 17β-oestradiol were quantitated by specific radioimmunoassays as described under Experimental. Points represent the averages of duplicate determinations: △, total oestrogens; ○, 17β-oestradiol; and ●, oestrone.

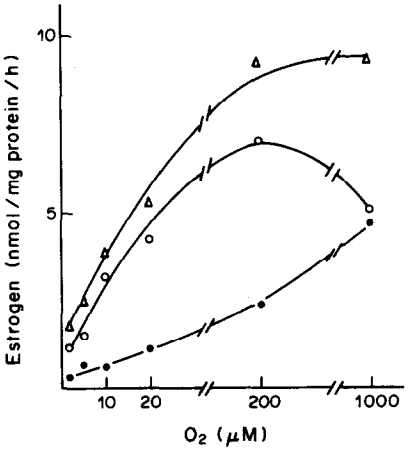

Fig. 3. Oxygen dependent aromatization of testosterone by placental microsomes. Conditions were as described in the legend to Fig. 2 except the substrate was 0.37 μM testosterone. Values represent the averages of 4 individual experiments: △, total oestrogens; ○, 17β-oestradiol; and ●, oestrone.

These results suggested that the proportions of 17β-oestradiol and oestrone produced by the aromatase of placental micromes under the conditions described may be determined by the NADPH/$NADP^+$ ratio. Variations of the NADPH/$NADPH^+$ ratio under conditions where total oestrogen production was constant showed that regardless of the substrate used, the relative proportions of 17β-oestradiol and oestrone produced were dependent on the NADPH/$NADP^+$ ratio (Table 1). A high concentration of $NADP^+$ favoured the production of oestrone while a high NADPH concentration favoured that of 17β-oestradiol. Moreover, addition of excess NADPH (10 mM) to a system in which the production of oestrone was expected to predominate, substantially shifted the quilibrium in favour of 17β-oestradiol production (Table 1, condition 5). Thus, in the microsomal system, the equilibrium for the interconversion of oestrone and 17β-oestradiol was a function of the ratio of oxidized to reduced forms of NADP.

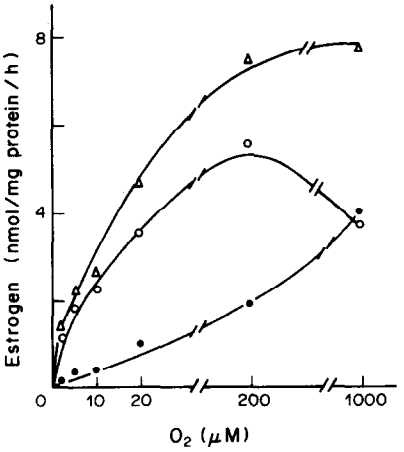

Fig. 2. Oxygen dependent aromatization of androstenedione by placental microsomes. Placental microsomes (0.2 mg protein/ml) were incubated in Kreb's–Henseleit buffer as described in the legend to Fig. 1. Incubations were carried out under various steady-state oxygen concentrations for 10 min using 0.37 μM androstenedione as substrate. Extraction and quantitation of oestrogens was as described for Fig. 1. Values represent the averages of 4 individual experiments: △, total oestrogens; ○, 17β-oestradiol; and ●, oestrone.

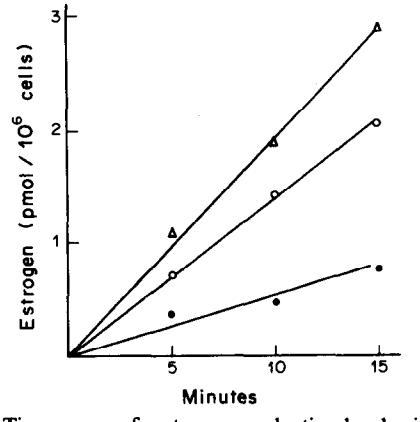

Fig. 4. Time course of oestrogen production by choriocarcinoma cells (BeWo line). BeWo cells (5×10^5/ml) were incubated in Ca^+-free Kreb's–Henseleit buffer containing 12.5 mM Hepes and 1 mM EDTA, pH 7.4, in rotating round-bottom flasks at 37°C. Reactions were initiated by addition of testosterone (0.37 μM) and allowed to proceed in air for the times specified. Extraction and quantitation of oestrogens was as described for Fig. 1. Values represent the averages of duplicate experiments: △, total oestrogens; ○, 17β-oestradiol; and ●, oestrone.

Table 1. Effect of the NADPH/NADP$^+$ ratio on aromatase activity of term human placental microsomes

Condition	NADPH/NADP$^+$ ratio	Products produced (nmol/mg protein) From testosterone Oestradiol	From testosterone Oestrone	From androstenedione Oestradiol	From androstenedione Oestrone	17β-Oestradiol/oestrone ratio Substrate added[a] Testosterone	Androstenedione
1. 5 mM NADPH	—	1.48	0.54	1.20	0.57	5.73 (2.74)	4.19 (2.11)
2. 2.5 mM NADPH 2.5 mM NADP$^+$	1.0	1.06	1.08	0.72	1.04	1.03 (0.98)	0.71 (0.69)
3. 1 mM NADPH 4 mM NADP$^+$	0.2	0.50	1.48	0.52	1.47	0.36 (0.34)	0.38 (0.35)
4. 0.5 mM NADPH 4.5 mM NADP$^+$	0.1	0.36	1.62	0.27	1.47	0.22 (0.22)	0.19 (0.18)
5. 0.5 mM NADPH 4.5 mM NADP$^+$ (+10 mM NADPH)	0.1	1.23	0.92	0.91	0.85	1.02 (1.32)	0.89 (1.07)

Microsomes were incubated at 37°C under air for 10 min with 0.37 μM testosterone or androstenedione as substrate. Reaction mixtures initially contained NADPH and NADP$^+$ contents as given. Under condition 5, the ratio of NADPH/NADP$^+$ was changed rapidly at 8 min by addition of 10 mM NADPH to test whether the ratio of product formation was sensitive to NADPH/NADP$^+$ ratio.
[a]Because total product was not the same for each experiment, ratios were calculated for individual experiments and then averaged. Values in parentheses are ratios calculated from the averages of product formation given in columns 3–6.

Studies on BeWo cells

Intracellular regulatory processes frequently alter the apparent response of enzymes in cells to changes in substrate concentration. Consequently, we sought to examine the O_2 dependence of oestrogen production in an intact cellular system. Because of the similarity to normal placenta (see Discussion), the human choriocarcinoma cell line was selected. The time course of formation (Fig. 4) shows that the production of oestrone, 17β-oestradiol and total oestrogens were linear for at least 15 min. Similar results were obtained using androstenedione as substrate (data not shown).

The oxygen dependence of aromatase activity of BeWo cells showed similar profiles for both androstenedione and testosterone as substrate (Figs 5 and 6, respectively) in that the production of total oestrogen (17β-oestradiol plus oestrone) was O_2-dependent over the entire O_2 concentration range. However, the O_2 dependencies of both 17β-oestradiol and oestrone formation were different with the two substrates. With testosterone as substrate, oestrone production saturated at a very low O_2 concentration (about 10 μM) while the production of 17β-oestradiol did not saturate even at 1 mM O_2. Since 17β-oestradiol was produced at about 3 times the rate of oestrone, total oestrogen production also did not saturate with respect to O_2 concentration. With androstenedione as substrate, neither 17β-oestradiol nor oestrone production saturated with O_2 concentration. Thus, the function of the aromatase in intact BeWo cells is markedly more sensitive to O_2 concentration than placental microsomal aromatase.

Because of the possibility that the BeWo microsomal aromatase differs from the placental microsomal aromastase, we also examined the O_2 de-

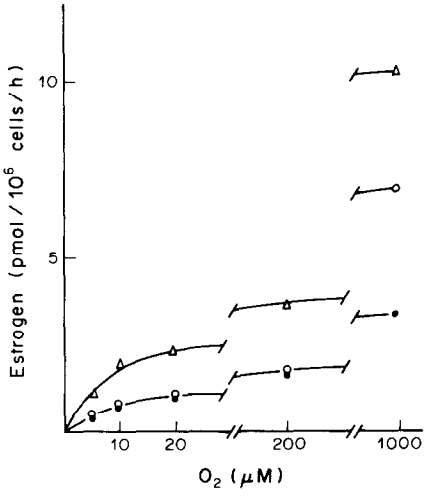

Fig. 5. Oxygen dependent aromatization of androstenedione by BeWo cells. BeWo cells were incubated in Kreb's–Henseleit buffer as described for Fig. 4. Incubations were performed under various steady-state oxygen concentrations for 10 min using 0.37 μM androstenedione as substrate. Extraction and quantitation of oestrogens was as described. Values represent the averages of 4 individual cell preparations: △, total oestrogens; ○, 17β-oestradiol; and ●, oestrone.

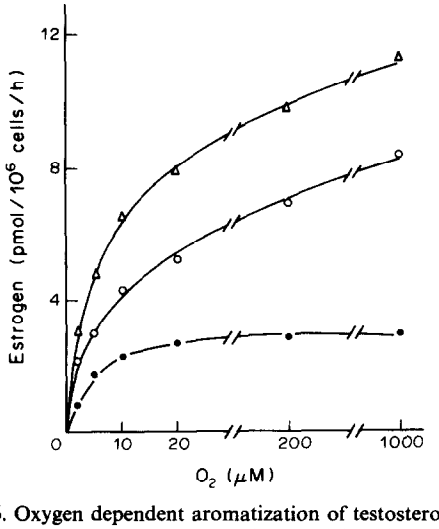

Fig. 6. Oxygen dependent aromatization of testosterone by BeWo cells. Conditions were as described for Fig. 5 except the substrate was 0.37 μM testosterone. Values represent the averages of 4 individual cell preparations; △, total oestrogens; ○, 17β-oestradiol; and ●, oestrone.

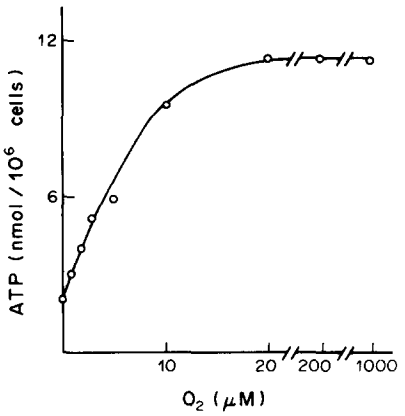

Fig. 7. ATP concentration in BeWo cells as a function of oxygen concentration. Cells were incubated as described in the legend to Fig. 5 with androstenedione as substrate. Following removal of protein by addition of perchloric acid (1 M final concentration) and centrifugation, samples were neutralized and analysed by HPLC as previously described [10].

pendence of BeWo microsomal aromatase. The results showed the same general O_2 dependence pattern as seen for the placental microsomes (data not shown). Therefore, the differences in O_2 dependence between cells and microsomes appears to be due to intracellular factors which modulate the aromatase activity rather than to differences between normal placental aromatase and BeWo aromatase.

The dependence upon O_2 concentration at such high values indicates that oestrogen synthesis may be selectively sensitive to oxygen deficiency. Since cells are primarily dependent upon the adequacy of O_2 supply for mitochondrial ATP production, we examined the O_2 dependence of ATP concentration in BeWo cells. The results (Fig. 7) show that ATP concentration does not decrease except below 25 μM O_2 and that a half-maximal change occurs at about 6 μM O_2. Thus, ATP supply in BeWo cells is relatively resistant to hypoxic O_2 concentrations as compared to the sensitivity of the aromatase.

DISCUSSION

Choriocarcinoma cells have been extensively characterized. They resemble the normal placenta with regard to morphology [12], enzyme activities [13] and the ability to synthesize hCG [14, 15] and steroid hormones [16–18]. These cells therefore provide a suitable cellular model for the study of oxygen-dependent processes of the placenta.

In the present studies, changes in the rates of oestrogen synthesis as a function of oxygen concentration were examined in isolated placental microsomes and in BeWo cells. Since 3 moles of O_2 are required per mole of product formed [5], the synthesis of oestrone and 17β-oestradiol is expected to be sensitive to oxygen concentration. The results show that in both whole cells and isolated microsomes, the rate of synthesis of oestrone and 17β-oestradiol were dependent on oxygen concentration.

In a previous study with placental microsomes, Zachariah and Juchau[7] found that the aromatization of androstenedione to oestrone was faster in the presence of 100% O_2 than in air. The present study with placental microsomes shows that the "half-maximal" rate of oestrone production occurs at a similarly high O_2 concentration although it should be noted that the reaction does not saturate even at 1 mM O_2. Since 17β-oestradiol was the major product of the reaction, inclusion of measurements of 17β-oestradiol shows that the half-maximal rate for *total* oestrogen production occurs at a much lower oxygen concentration, approx 14 μM O_2. In agreement with previous studies [19], it was also shown that the proportions of 17β-oestradiol and oestrone formed in a given incubation were dependent on the NADPH/$NADP^+$ ratio (see Table 1).

In the microsomal system the oxygen dependence profiles were similar for both androstenedione and testosterone as substrates. This finding suggests that in the presence of exogenous reductant, interconversion of products readily occurred. Regardless of the substrate, the major product of the reaction was 17β-oestradiol at all oxygen concentrations except 1 mM, where the rate of oestrone production increased relative to that of 17β-oestradiol. Since the non-substrate dependent autooxidation of NADPH is nearly linear with increasing oxygen concentration [20], this result may be explained by a change in the NADPH/$NADP^+$ ratio of the incubation.

The oxygen-dependent production of oestrogens by whole cells differed from that observed with isolated microsomes. With cells, 17β-oestradiol was the major product at all oxygen concentrations. 17β-Oestradiol and total oestrogen production continued to increase even at 1 mM O_2. Furthermore, a reversed pattern of oestrone to 17β-oestradiol synthesis did not occur at 1 mM O_2, which is consistent with the intracellular environment being relatively reduced [21, 22].

The differences between the O_2 dependence of cells and microsomes for total oestrogen production is in marked contrast to previous results for hepatic cytochrome P-450 which show that microsomes and isolated liver cells have the same O_2 dependences [23]. The unusually high oxygen dependence for oestrogen synthesis in BeWo cells is important because it indicates that oestrogen biosynthesis *in vivo* may also be O_2 dependent. Comparison to the O_2 dependence of energy metabolism, as indicated by the ATP concentration (Fig. 7), shows that oestrogen production is considerably more sensitive to hypoxia.

The nature of the factor(s) responsible for the dependence of the aromatase on oxygen in cells is not known. Since the concentration of oestrone did not increase at oxygen concentrations above 10 μM, oestrone may act in some manner as a feedback in-

hibitor. Alternatively, regulation of the aromatase by a phosphorylation/dephosphorylation mechanism has been proposed [1] and this may function in altering the enzymatic characteristics of the aromatase in the cells.

The possibility that placental oestrogen biosynthesis is sensitive to oxygenation has important implications for a variety of physiological and pathological conditions. Prolonged strenuous exercise, anemia, ventilatory insufficiency and exposure to low barometric pressures reduce arterial oxygenation and consequently, may directly decrease the oxygen supply to the placental aromatase. In addition, decreased oxygen-carrying capacity of blood due to formation of carbon monoxyhemoglobin, as occurs in cigarette smokers, could lower placental oxygenation. A decrease in placental oxygenation may result in impairment of steroid hormone production, particularly oestrogens, and therefore to a decrease in the functional state of the placenta. However, additional studies will be required to completely understand the complex effects of hypoxia on oestrogen biosynthesis and to examine its importance *in vivo*.

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