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Human Cytochrome *c* Oxidase During Cardiac Growth and Development

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SUMMARY. **Human heart mitochondrial cytochrome *c* oxidase specific content and specific activity was measured in five fetuses 15–21 weeks gestational age and in five patients whose age ranged from 6 days to 22 years. None had evidence of cardiac pathology. An increase in cytochrome *c* oxidase specific content and specific activity was observed in the fetal heart with increasing gestational age (0.13–0.38 nmol heme *a*/mg protein and 67–295 nmol O_2 utilized/min/mg protein) and from the neonatal period (0.35 nmol heme *a*/mg protein and 140 nmol O_2/min/mg protein) to adulthood (1.2 nmol heme *a*/mg protein and 1104 nmol O_2/min/mg protein). A marked increase was observed postnatally between 4 and 19 months.**

KEY WORDS: **Cytochrome *c* oxidase — Mitochondria — Human heart — Fetal heart**

Cytochrome *c* oxidase (ferrocytochrome c: oxygen oxidoreductase, E.C. 1.9.3.1) is the terminal enzyme of the mitochondrial respiratory chain. It is intrinsic to the mitochondrial inner membrane and functions to transfer four electrons from reduced cytochrome *c* to oxygen. Under coupled conditions, the free energy released in this reaction is converted to a proton gradient potential (by pumping out proton ions across the inner membrane) that may be used by the ATP synthetase complex to make ATP from ADP and inorganic phosphate and to break down the gradient [7]. Kadenbach [9] has recently reviewed the experimental evidence that the enzyme's catalytic activities are affected by allosteric modification, and suggested that respiration and ATP synthesis in higher organisms are regulated by allosteric modification of respiratory chain complexes, in particular, of cytochrome *c* oxidase. Kuhn-Nentwig and Kadenbach [15] have presented evidence that some of the nuclear-coded subunits of the enzyme are different in fetal and adult rats.

Early myocardial cells of the rat embryo heart and other animal models show evidence for functional mitochondrial oxidative phosphorylation and electron transport enzymes, but low specific activity and specific content on a mitochondrial and homogenate protein basis compared to late gestation [8, 17, 21, 23, 27]. Whereas in rat heart, cytochrome *c* oxidase activity and content increased from the neonatal period to adulthood [4, 5, 14, 21, 25], in other mammals conflicting results have been reported [28, 31]. Wells et al. [28] reported 56 and 65% greater cytochrome *c* oxidase activities in fetal and newborn heart mitochondria, respectively, when compared with the adult sheep. In contrast, Young et al. [31] found no difference in cytochrome *c* oxidase activity between newborn and adult New Zealand white rabbits. Smith and Page [20] determined mitochondrial ultrastructural changes in left ventricular myocardial cells from New Zealand white rabbit during the interval from 3 days before to 4 days after birth. This interval was associated with a rapid and large accumulation of mitochondria and myofibrils, and mitochondria also were found to be packed more densely with cristae. In view of the conflicting results in mammalian hearts and the lack of information about the influence of maturation on human heart cytochrome *c* oxidase and its relationship to mitochondrial development, this study was initiated utilizing normal fetal and postnatal hearts to provide some initial baseline data on the cardiac maturation of the activity and content of this vital cardiac enzyme in man.

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Materials and Methods

Sample Harvesting and Handling

Fetal heart samples were obtained from second trimester fetuses (15–21 weeks gestational age) *immediately* following elective abortion and transferred *immediately* to the laboratory on ice. Postnatal human heart samples were obtained at autopsy from patients dying of noncardiac causes with 4–6 h postmortem except 20 h in the patient aged 22 years. Bodies were kept at 4°C until processed. The samples were put in isotonic saline solution on ice and transported to the laboratory. All subsequent manipulations were carried out at 4°C.

Keilin-Hartree Preparation

Mitochondrial inner membrane for cytochrome *c* oxidase measurements was prepared by a modified Keilin-Hartree procedure [13, 26] initiated within 1 h after harvesting the heart. This procedure removes hemoglobin and myoglobin which interfere with cytochrome spectral analysis. The preparation contains a complete set of the enzymes capable of oxidation of succinate and NADH by dioxygen to fumarate and NAD, respectively, and water [26].

Fetal samples were minced into ~2-mm pieces and washed by centrifugation at 2000 rpm for 10 min in a Sorvall SS-34 rotor with 0.1 M potassium phosphate–0.1 mM EDTA (KPE) until the redness turned to pale yellow. This was followed with a distilled-water wash. Postnatal ventricular myocardium samples were cleaned of all connective and fatty tissue, minced into ~2-mm cubes, and washed in distilled water 30 min and overnight in KPE to wash out any hemoglobin and myoglobin. In the morning the sample was washed with distilled water for 30 min. Between washes the sample was filtered over cheese cloth and resuspended. After washing, the preparation was suspended in a minimal amount of 0.25 M mannitol–20 mM TrisHCl–1 mM EDTA, pH 7.4 (MTE), and the resulting "brie" was homogenized in a small teflon/glass homogenizer.

The heart homogenates were centrifuged at 2500 rpm for 10 min in a Sorvall SS-34 rotor to pellet whole cells, tissue, and cell debris. The pellet was resuspended in MTE, rehomogenized, and recentrifuged at 2500 rpm for 10 min in the SS-34. The supernatants from the first spin and the wash were combined and centrifuged at 21,000 rpm (53,000 *g*) for 25 min in a Beckman 50.2 Ti rotor to pellet the inner mitochondrial membrane [26]. The fresh inner mitochondrial membrane pellet was resuspended in a small volume of MTE buffer and assayed for protein, cytochrome content (heme *a*), and cytochrome *c* oxidase activity.

Assays

Cytochrome *c* oxidase (heme *a*) concentrations were determined on an Aminco DW-2a dual-beam spectrophotometer from the dithionite-reduced minus ferricyanide-oxidized 605 nm minus 630 nm difference spectra of isolated mitochondria at room temperature using an extinction coefficient of 10.4 mM [24]. Cytochrome *c* oxidase activity was assayed polarographically with a Clark oxygen electrode according to a method adopted from Ferguson-Miller et al. [2, 3], as the rate of oxygen uptake at 25°C in the presence of tetramethylphenylenediamine and ascorbic acid as reducing agents, and a saturating dose (31 μM) of cytochrome *c* in a medium of 25 mM Tris-acetate–250 mM sucrose–0.25% Tween 80, pH 7.9. About 20–60 μg protein was used. The difference in rates obtained before and after the addition of cytochrome *c* was taken as the enzyme-specific activity (nmol O_2 utilized/min/mg protein). Protein was determined according to the method of Lowry et al. [16], using crystalline bovine serum albumin as a standard. Samples were prepared for electron microscopy according to the procedure of Smith and Page [19] after removing a small piece of the left ventricle from fetal or postnatal heart specimens, and fixing it in a fresh solution of 2% gluteraldehyde-0.1 M cacodylate buffer, pH 7.4.

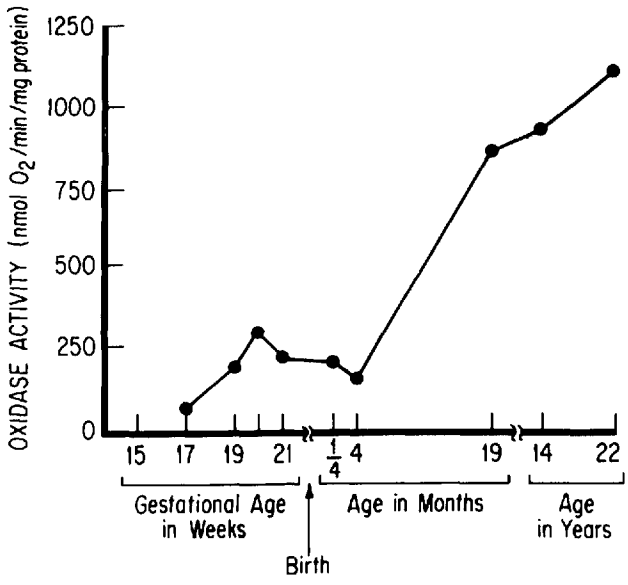

Fig. 1. Plot of human heart cytochrome *c* oxidase specific activity (nmol O_2 consumed/min/mg protein) vs age, including second trimester prenatal data.

Results

Figure 1 shows that cytochrome *c* oxidase specific activity (nmol O_2/min/mg mitochondrial protein) increased markedly between 4 (140 U) and 19 months (863 U), and continued to increase somewhat through adolescence to adulthood. At 22 years of age 1104 U were measured. The oxidase concentration (nmol heme *a*/mg mitochondrial protein) increased more or less continuously (Fig. 2) from 6 days of age (0.35 U) through adolescence to adulthood, and at 22 years of age 1.2 U were measured. The fetal data (Fig. 1) obtained during the course of the second trimester showed an increase in cytochrome *c* oxidase specific activity from 67 nmol O_2 uptake/min/mg protein to about 295 nmol O_2 uptake/min/mg protein from 15–21 weeks gestational age. The cytochrome *c* oxidase content (Fig. 2) also increased from 0.13–0.38 nmol heme *a*/mg protein. Electron microscopy of fetal heart samples showed

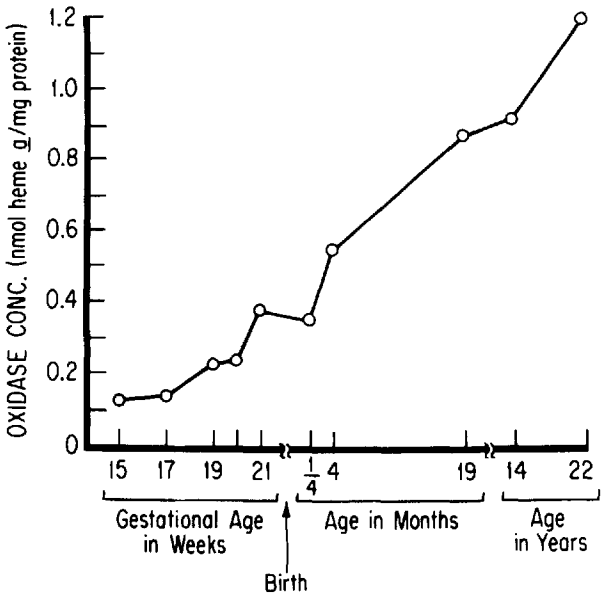

Fig. 2. Plot of age vs cytochrome *c* oxidase specific content (heme *a*/mg protein), including second trimester prenatal data.

fewer mitochondria with less matrical density and sparse cristae than in the postnatal sections (unpublished observations).

Discussion

Cytochrome *c* oxidase is an integral protein of the mitochondrial inner membrane and plays an essential role in cellular respiration. The enzyme has been extensively studied in aerobic bacteria, lower eukaryotic organisms, and mammals [7]; however, little is known about this enzyme in humans. Studies on human placenta and HeLa cells demonstrated that the three mitochondrially derived human cytochrome *c* oxidase subunits (I–III), which display the catalytic activity of the enzyme (i.e., electron transport from cytochrome *c* to oxygen and proton translocation), showed a high degree of homology to other eukaryotic cytochrome *c* oxidases [6]. Although their function is uncertain, there are 10 cytoplasmically derived subunits in the mammalian form of the enzyme [10–12, 18, 22]. Kadenbach's group has presented evidence for a tissue-specific role of some of the nuclear-coded polypeptide subunits in the cytochrome *c* binding domain of the mammalian enzyme, and has also hypothesized a regulatory role for some of the nuclear-encoded subunits [10, 22]. It has been demonstrated in yeast that subunit IV, which is nuclear-coded and cytosol-synthesized, is necessary for cytochrome *c* oxidase assembly [1].

In this report we have presented data on the marked increase between fetal and postnatal human heart cytochrome *c* oxidase specific activity and specific content (measured as heme *a* content). We were concerned about using postmortem heart material and attempted to minimize these effects. The fetal specimens were harvested immediately following elective abortion and transferred on ice to the laboratory. With postnatal heart samples we believe the 4- to 6-h time frame did not lead to significant change in cytochrome *c* oxidase. The observed trend extended through 14 years of age. Although the heart tissue in the 22-year-old patient was obtained 20 h postmortem, our experience with rat heart (unpublished observations) shows that the enzyme is stable even after 24 h of cold storage.

Although some functioning mitochondria are present during the early embryonic stages of a variety of eukaryotic organisms that use oxygen for respiration (including mammals), relative to adult myocardial cells, fetal heart mitochondria function at a very low oxygen level [21, 27]. Postnatally, it has been shown that both morphological and biochemical changes occur in mammalian heart mitochondria [20, 25]. This has been attributed to the transition from a hypoxic environment of the fetus to a more aerobic environment of the newborn [5]. Other explanations may be a change in the protein makeup of fetal as compared to postnatal forms of the mitochondrial enzymes accompanying a metabolic shift from carbohydrates to fatty acids as the predominant energy source [15, 27, 29]. Alternatively, the increased mitochondrial cytochrome *c* oxidase specific content (measured as heme *a* spectrally) and specific activity observed with human cardiac maturation may arise from the heme-*a*-induced sequential assembly of the enzyme's subunits within the mitochondrial inner membrane [30]. Further investigation is necessary to establish the molecular mechanism for the results of this report of dramatically increased levels of cytochrome *c* oxidase during cardiac maturation. One approach is to determine the levels of immunoreactive cytochrome *c* oxidase subunits in isolated fetal and postnatal heart mitochondria.

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