Vol. 111, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1983 Pages 424-429

EFFECTS OF HUMAN CHORIOGONADOTROPIN ON MITOCHONDRIAL AND MICROSOMAL CYTOCHROME P-450 LEVELS IN MOUSE TESTES[1]

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Received January 18, 1983

SUMMARY: The mitochondrial and microsomal cytochrome P-450 contents of C57B1/6 mouse testis have been measured using difference spectroscopy on stable enzyme preparations containing the ferrous-carbon monoxide complex. Results were obtained on control animals (52 ± 3 days of age) and on animals injected subcutaneously with human choriogonadotropin (0.017 μg/g body weight 24 h prior to sacrifice). The high ratio of testicular mitochondrial cytochrome oxidase to P-450, which has previously precluded measurements of basal P-450 levels, was overcome by using N,N,N',N'-tetramethyl-p-phenylene diamine to bypass site II, in combination with antimycin A to prevent reverse electron flow. The basal levels of mitochondrial and microsomal P-450 in mouse testis were 37.9 ± 3.5 and 28.9 ± 1.6 pmol/mg protein, respectively. Following administration of a desensitizing dose of gonadotropin, the respective values were lowered to 19.9 ± 1.4 and 19.6 ± 2.1 pmol/mg protein in 24 h. This is the first report of a gonadotropin-mediated decrease in mitochondrial P-450 and thus demonstrates that desensitization leads to alterations in both microsomal and mitochondrial P-450 in mouse testis.

Cytochrome P-450 plays an important role in steroidogenesis and consequently is the focus for a number of studies in trophic hormone target tissues such as the adrenal cortex (1), ovaries (2-4), and testes (5). In testes, the microsomal enzyme from porcine neonates has been purified and characterized by Nakajn et al. (6,7). Interestingly, they found that the microsomal enzyme contained both 17α-hydroxylase and $C_{17,20}$ lyase activity (8). Nozu et al., working with adult male rats, showed that the microsomal content of P-450 was substantially lower in animals treated with a desensitizing dose of hCG (9).

1) Portions of this work were presented at the 64th Annual Meeting of The Endocrine Society, San Francisco, CA, June 16-18, 1982, abstract no. 42.

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Abbreviations: hCG, human choriogonadotropin; P-450, cytochrome P-450; PG, 0.1 M potassium pyrophosphate (pH 7.4) containing 30% (v/v) glycerol; TMPD, N,N,N',N'-tetramethyl-p-phenylene diamine; TS, 20 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose, 20 mM KCl, and 1 mM EDTA.

0006-291X/83 $1.50

In addition, they found the enzyme to be quite unstable in the microsomal fraction (9).

The mitochondrial side chain cleavage enzyme from rat testis, unlike that from adrenals, has proven difficult to study since the relatively high level of cytochrome oxidase obscures the 450 nm absorption peak in spectrophotometric assays, prohibiting accurate measurements of levels (5).We have also found a relatively high ratio of cytochrome oxidase to P-450 in mouse testis. In order to study changes in the levels of the side chain cleavage enzyme with hCG treatment, a method has been used which permits an accurate determination of P-450 without interference from cytochrome oxidase (10). This technique involves standard difference spectroscopy of the reduced P-450 carbon monoxide complex by bypassing site II with N,N,N',N'-tetramethyl-p-phenylene diamine and preventing reverse electron flow using antimycin A. With this method we have been able to measure basal levels of mouse testicular mitochondrial P-450 and changes 1 day following a desensitizing dose of hCG. The corresponding levels of microsomal P-450 in mouse testis and the effects of a desensitizing dose of hCG on this enzyme were also measured.

MATERIALS AND METHODS

Desensitization and preparation of a mouse Leydig cell-enriched fraction. C57Bl/6 male mice (about 18 g body weight) were obtained from Jackson Laboratories, Bar Harbor, MA, and sacrificed between 49 and 55 days of age via cervical dislocation. Desensitization was accomplished with 0.3 μg of purified hCG (11) in 0.1 ml phosphate-buffered saline given subcutaneously 24 h prior to sacrifice, while control animals received only buffer. Decapsulated testes were placed in ice-cold 20 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 20 mM KCl, and 1 mM EDTA (TS). A section of each testis was removed to prepare a Leydig cell fraction, nonenzymatically as described by Schumacher et al. (12) for an in vitro primary cell culture. Cells were incubated in Linbro culture wells for 3 h at 37°C under a humidified atmosphere of 95% air/5% CO_2. The final volume of 1 ml contained 10^6 cells, 1 mg of bovine serum albumin, and 0.1 mg gentamycin in Medium 199. The medium was assayed for testosterone by radioimmunoassay.

Preparation of testicular mitochondrial and microsomal fractions. Decapsulated testes, in 10X volume of TS, were homogenized with a Potter-Elvehjem Teflon-glass homogenizer. The homogenate was centrifuged at 3000xg for 15 min, and the supernatant was recentrifuged at 9000xg to obtain a mitochondrial pellet. This was washed twice (once with TS and once with TS minus sucrose), and the final pellet was resuspended in 0.1 M potassium pyrophosphate, pH 7.4 (9), containing 30% (v/v) glycerol (PG). The supernatant from the first 9000xg centrifugation was centrifuged at 100,000xg for 1 h, the pellet was washed once in TS minus sucrose to reduce hemoglobin contamination (13), and the final pellet was resuspended in PG. All the above operations were conducted at 4°C. Protein determinations on the subcellular fractions were performed by the

method of Lowry et al. (14) in the presence of 1% deoxycholate with bovine serum albumin as standard.

Measurement of testicular and tumor mitochondrial and microsomal P-450. Quantification of the testicular mitochondrial enzyme was performed by analysis of the CO difference spectrum using a variation of the method of Ghazarian et al. (10). Mitochondria were first diluted to 20 mg protein/ml with PG and then 1:1 with 30 mM triethanolamine in 30% PG. Equal aliquots (0.2 ml) of this mitochondrial suspension were placed in microcuvettes. The following additions were made to the reference cell in order: 10 µL of 10 mM antimycin A in 95% ethanol, 25 µL of 0.1 M ascorbate (pH 7.0), and 25 µL of TMPD (Sigma Chemical Co., St. Louis, MO). The sample cell received, in order, the following: 10 µL of 95% ethanol, 10 µL of 0.1 M malate (pH 7.0), 25 µL of 0.1 M ascorbate (pH 7.0), and 15 µL H_2O. A baseline was established by bubbling N_2 through both cuvettes. Next, CO was bubbled through the cuvettes, and the difference spectrum between 400-500 nm was recorded using a Cary 210 spectrophotometer with a spectral band width of 3.5 nm.

Testicular microsomal P-450 was measured as described by Nozu et al. (9) with the following modifications. The solubilization buffer was that described by Guengerich (15), and microcuvettes were used. The mitochondrial and microsomal P-450 contents of the Leydig cell tumor were quantitated as described by Omura and Sato (16). An extinction coefficient of 91 mM^{-1} cm^{-1} was used for all P-450 preparations (16).

RESULTS

The standard CO difference spectrum of mouse testicular mitochondrial P-450 is difficult to resolve because of the significant contribution of cytochrome oxidase at 420 nm (Fig. 1A). The use of the TMPD bypass technique alleviated this interference and permitted rapid measurement of the mitochondrial P-450 component, which is characterized by a peak at 448 nm and an isosbestic point at 464 nm (Fig. 1B).

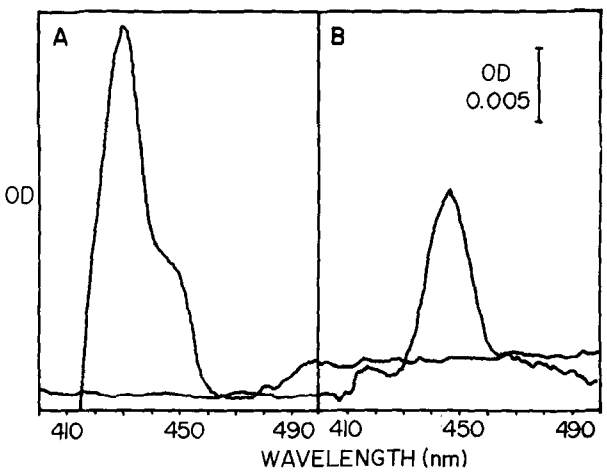

Figure 1. CO difference spectra of mouse testicular mitochondrial P-450. (A) Standard assay conditions. The major contribution of cytochrome oxidase at 420 nm obscures the 450 nm component. (B) Assay using the TMPD bypass as described in the text.

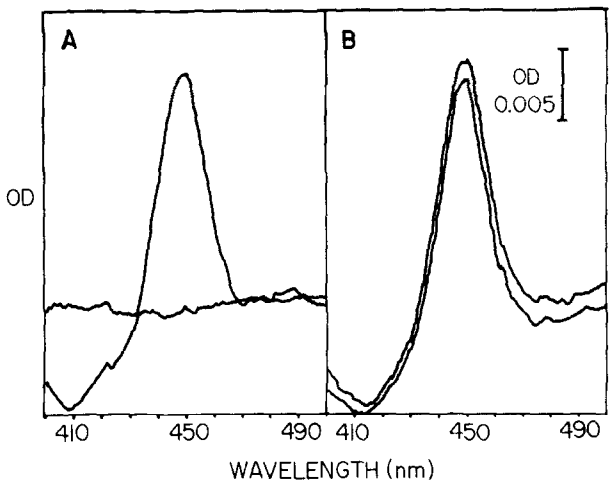

Figure 2. CO difference spectra of mouse testicular microsomal P-450. (A) Difference spectrum immediately after addition of sodium dithionite. (B) Repeat scans at 10 min (lower curve) and 20 min (upper curve) after dithionite reduction.

The mouse testicular microsomal P-450 content can be measured directly, and the resulting preparation was stable with time as repeat scans at 10 min and 20 min were not significantly different (Fig. 2). In addition, these preparations were stable to freeze-thawing (data not shown). This behavior is in contrast to that reported for a microsomal P-450 fraction from rat testis (9).

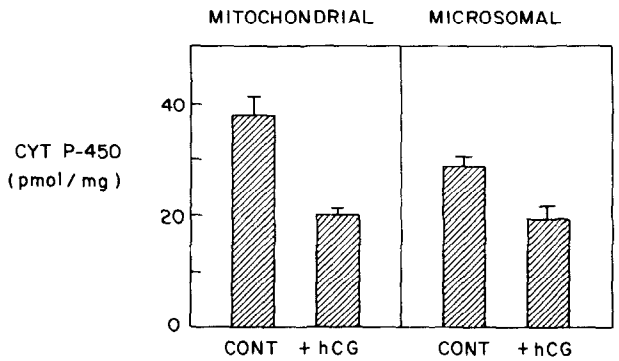

Figure 3. Mitochondrial and microsomal P-450 levels (units of pmol/mg protein) in mouse testes as determined using the spectral assays described in Figs. 1 and 2, respectively. The values from control animals (CONT) are based on 4 separate experiments, and those from animals treated with a desensitizing dose of hCG(+hCG) are derived from 3 separate experiments. Typically, each experiment used pooled testes from 12 animals. The results are shown as mean + SE.

The basal levels of mouse testicular mitochondrial and microsomal levels of P-450 are presented in Fig. 3. Following a desensitizing dose of hCG, the levels of P-450 have dropped significantly in 24 h (Fig. 3). Treatment with hCG led to a 47% decrease in mitochondrial P-450 while microsomal P-450 dropped 32% in comparison to controls. Desensitization was confirmed by measuring the testosterone production in response to a maximally stimulatory dose of hCG by Leydig cell-enriched fractions from control and hCG-treated animals. Following an *in vitro* incubation for 3 h (control cells and cells plus hCG), the per cent increase over basal of testosterone production by cells from treated mice was only about 15% that of cells from control mice.

DISCUSSION

Using the TMPD bypass of site II of oxidative phosphorylation, in combination with antimycin A to prevent electron flow (10), we have been able to measure for the first time basal levels of mouse testicular mitochondrial P-450. Of interest is the observation that the levels of both mitochondrial and microsomal P-450 are significantly decreased 1 day after administration of a desensitizing dose of hCG. Our results on the hCG-mediated decrease in the content of the microsomal enzyme confirm in mouse testis the report by Nozu *et al.* on rat testis (9). It should be pointed out, however, that our enzyme preparations exhibited reasonable stability, unlike that of the rat microsomal P-450 which began converting to a 420 nm form immediately upon addition of sodium dithionite (9). Indeed, our microsomal preparations were stable with time, at least up to 30 min after addition of sodium dithionite, and to freeze-thawing.

The present results with mouse testis suggest that one lesion in gonadotropin-mediated desensitization of Leydig cells may be a reduction in the content of the mitochondrial cholesterol side chain cleavage enzyme. Thus, mitochondrial P-450 may represent a key regulatory site under the influence of LH that precedes the microsomal enzymes, also under gonadotropin regulation.

ACKNOWLEDGMENTS

We thank Sharon Tollefson for expert technical assistance and Dr. Philip Wang for helpful discussions. This research was supported by the NIH:

Research Grants AM15838 and ES01590; Training Grants HD07043(JDL) and CA09131(MHM); and Center Grant HD05797 which provided cell culture facilities.

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