Vol. 173, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1990 Pages 302-308

SUPEROXIDE DISMUTASE AS A REGULATORY SWITCH IN MAMMALIAN TESTICULAR STEROIDOGENESIS

Pradeep Kumar G., Neeta Seerwani, Malini Laloraya,
Manish Nivsarkar, Saguna Verma and Aparna Singh

School of Life Sciences,
Devi Ahilya Vishwavidyalaya,
Indore- 452 001, India

Received October 15, 1990

The Δ^4-pathway of testosterone biosynthesis in leydig cells, widely believed to proceed through pregnenolone-->pregnenedione-->progesterone route catalyzed by Δ^5-3β-hydroxysteroid dehydrogenase and Δ^5-Δ^4-isomerase respectively is shown to pass through an alternate pathway mediated by superoxide dismutase and peroxidase. A built-in regulatory switch is incorporated in this route, with the superoxide dismutase inducible upon LH-stimulation of the leydig cells.

The conversion of Δ^5-pregnenolone to testosterone in the testis can occur along several metabolic pathways (1-3). The two main biosynthetic routes are identified; one of them is the so called Δ^4-pathway: pregnenolone--> pregenenedione--> progesterone--> α-hydroxyprogesterone--> androstenedione--> testosterone. The other is the Δ^5-pathway: pregnenolone-->α-hydroxy-pregnenolone-->dehydro-epiandrosterone-->androstenediol-->testosterone. Species differ with regard to the relative importance of the two pathways (4). Evidences document that 5α-reduced androgens are major products of interstitial tissue in the rat from the time of weaning upto the pubertal increase in testosterone production (5). The conversion of pregnenolone to testosterone involves several enzymes, some of which depend on oxygen and mitochondrial cytochrome P-450 for complete activity; a high proportion of the rat testicular cytochrome P-450 is concentrated in the microsomes of interstitial tissue (6,7). The two enzymes participating in the conversion of pregnenolone to progesterone are (i) the NAD-dependent Δ^5-3β-hydroxysteroid dehydrogenase present in the leydig cells and seminiferous tubules and converting Δ^5-pregnenolone to the corresponding

0006-291X/90 $1.50

seminiferous tubules and converting Δ^5-pregnenolone to the corresponding ketosteroid i.e., Δ^5-pregnenedione; and (ii) the Δ^5-Δ^4-isomerase (probably in the form of several isoenzymes), which catalyses the shift of the double bond from the Δ^5 position in pregnenedione to the Δ^4 position in progesterone. In addition to the enzymes listed above, another enzyme, viz., peroxidase has been shown to mediate the conversion of pregnenolone to progesterone through a free-radical mechanism coupled with ascorbate-semidehydroascorbate interchange. Recent studies have shown that LH induces peroxidase (8) and superoxide dismutase (9), thus regulating luteal steroidogenesis by switching on/off a multicomponent oxidase-peroxidase system with superoxide dismutase as one of its components. But an absolute lack in the literature regarding the operation of homologous enzyme pathways leading to the conversion of pregnenolone to progesterone in the leydig cells is identified, and this study is a pioneer attempt directed to attempt this issue.

MATERIALS AND METHODS

Reagents. Trizma base, Trizma HCl, Triton X-100, Diethyl dithio- carbamic acid (sodium salt), N-2-hydroxy ethyl piperazine-N'-2-ethane sulphonic acid, diethylenetriaminepentacetic acid and lutenizing hormone were obtained from Sigma Chemical Co., USA. N-t-butyl-α-phenyl nitrone was obtained from Aldrich, Milwaukee, WI. Pyrogallol was from Loba Chemie, Indo-Austranal, India. Tris-HCl buffer (50mM, pH 8.2) was made by mixing 50mM trizma base and 50mM trizma HCl in the ratio of 2:1. 1mM DTPA was added to this solution and the pH was adjusted to 8.2 with 50mM HEPES at 27°C.

Animals Mature male mice (Swiss strain) of the age group 2-3 months, inbred in our departmental stock house and maintained under a strict temperature (27±1°C) and light (14h light : 10h dark) regimen with food and water provided ad libitum were used for studies involving superoxide dismutase assays and superoxide radical estimation. Immature mice (< 1 month old) were used for studies on LH triggered SOD induction.

Methods

1. LEYDIG CELL PREPARATION

Animals were sacrificed by cervical dislocation. Testis were excised and decapsulated before incubation in Hank's balanced salt solution (HBSS) containing 0.5 mg/ml collagenase, according to Bellve et al (1977) (10). The supernatant cells were decanted and washed in HBSS before further purification. This single cell suspension contained Leydig cells (15%-20% of the total cells), erythrocytes and other interstitial cell types, as well as small number of sertoli cells (< 1% of total cells). Purified leydig cells were obtained from these suspensions according to Schumacher et al (1978) (11), except that 10-80% ficoll discontinuous gradient were established in 15 ml glass corning tubes. Greater than 10^8 total cells could be loaded onto single 15 ml gradient. Leydig cells were recovered from 50%-60% interface, diluted, washed thoroughly and adjusted to a final concentration of 10^6 cells/ml in HBSS before further analysis.

2. ADMINISTRATION OF LH INTO IMMATURE MICE

For experiments with LH, immature (< 1 month old) male mice were given a subcutaneous injection of purified 20ug luteinizing hormone dissolved in 1 ml of physiological saline (0.9 % sodium chloride). Controls were designed which received similar injections minus LH. These mice were sacrificed after different time periods of LH-injection viz., 0, 15, 30, 60, 120 and 240 minutes. The leydig cells were collected as described earlier and processed for subsequent analysis.

3. SUPEROXIDE DISMUTASE ACTIVITY ASSAY

The leydig cells were suspended in 4ml of Tris-HCl buffer (50mM, pH 8.2) containing 0.1M DTPA and were homogenized at 4°C at a speed of 13,000rpm (3 cycles, 30 seconds each) using a Polytron homogenizer with PT10 accessory. The homogenates were treated with 0.2% Triton X-100 (final concentration) for 20 minutes at 4°C. After this treatment, the suspensions were centrifuged at a speed of 15,000rpm at 4°C using a Sorvall OTD 65B Ultracentrifuge and a T 865.1 fixed angle rotor. The pellets were discarded and the supernatants were assayed for SOD activity by the method of Marklund and Marklund, 1974 (12), using the ability of the enzyme to inhibit the autoxidation of pyrogallol in presence of a metal chelator, viz., DTPA. The enzyme kinetics was monitored on an LKB Ultrospec 4050 spectrophotometer equipped with peripheral data acquisition system. All calculations were made on per milligram fresh weight.

4. SPIN-TRAPPING OF SUPEROXIDE ANION RADICAL

The leydig cells were dispersed in HBSS at 2,500 rpm (2 cycles, 10 seconds each) using a Polytron homogenizer with PT10 accessory. The suspension was incubated with 50mM PBN and 1 x 10^{-3} M DDC (final concentrations) for 1 hr. at 27°C, After incubation, 25ul aliquots were transferred into glass capillaries (Clinicon International, GmbH) and one end flame sealed taking care not to warm the suspensions. The superoxide anion radical was detected by incorporating an inhibitor of SOD, viz., DDC in the incubation medium and trapping the radical as a PBN-adduct. The EPR spectra of the PBN-superoxide radical adduct was recorded on a Varian E-104 EPR spectrometer equipped with TM_{110} cavity. The instrument settings employed were: scan range- 100G, field set- 3237G, temperature- 27°C, time constant- 0.5sec., scan time- 8min., modulation amplitude- 2G, modulation frequency- 100kHz, microwave power- 5mW, microwave frequency- 9.01GHz and receiver gain- 2.5 x 10^4 x 10. The EPR absorption line intensities of the low, mid and high field lines were calculated (9) and were analyzed.

DATA ANALYSIS

All the above said experiments were repeated 5 times. Statistical analysis were performed using Introductory Statistics Software Package, version 1.0 (13). The degree of variance of the observations obtained was tested by subjecting them to one-way ANOVA.

RESULTS AND DISCUSSION

This study was undertaken with a preview that the steroidogenic machinery in the leydig cells of mammals could be utilizing free-radical mediated steroid interconversions with the active involvement of oxy radical species and the superoxide-dismutating enzymes. Choosing mice as model system, we have performed assays for the total superoxide dismutase activity and the superoxide-generation capacity of these cells. Also, the possibility of the modulation of superoxide dismutase activity by pituitary hormones was tested employing prepubertal mice primed with lutenizing hormone.

TABLE 1

Showing the superoxide dismutase activity and the superoxide anion radical generation by the isolated leydig cells of Mus musculus

Parameter under study	No. of replicates	Quantity ±s.e.m.
Superoxide dismutase activity (in immature mice)	3	$1.80 \pm 0.13 \times 10^{-2}$ (units/mg fresh weight)
Superoxide dismutase activity (adult mice)	3	$140.15 \pm 2.13 \times 10^{-2}$ (units/mg fresh weight)
$O_2^{\cdot -}$-PBN adduct spectral intensity (adult mice)	4	$49.18 \pm 1.32 \times 10^{-10}$ (arbitrary units/mg fr.wt)

The pertinent finding that has come up in this study is that the leydig cells, the steroidogenic cells in the male gonad, show remarkably good superoxide dismutase activity during the pubertal phase of their life, but the pre-pubertal superoxide dismutase levels in these cells appeared to be very low (Table 1). A pubertal increase in the superoxide anion radical generating capacity of the leydig cells also was very clear (Figure 1).

The changes in superoxide dismutase activity in the leydig cells after luteinizing hormone administration in pre-pubertal mice is shown in Figure 2. No statistically significant increase from the original levels of superoxide dismutase activity was observable in leydig cells at 15 minutes after LH-injection, which was elevated to its peak value ($p<0.01$) at 30 minutes post-injection. Then onwards, there was a gradual fall in superoxide dismutase activity, which tended to revert back to its original levels. The phenomenon observed at 15 minutes and 30 minutes could not be traced in control experiments, in which the animals received an equal volume of physiological saline instead of LH.

In most mammals, the leydig cells resemble each other closely in ultrastructure and hormonal activity, and their growth and development pattern exhibits at least two distant phases or waves, one occurring in the fetus and another at the time of male puberty. The existence of a biphasic pattern, originally established in the rat has been amply confirmed and extended by biochemical and histochemical observations (14). Another interesting aspect in leydig cell steroidogenesis is that it can follow either a "Δ^4-pathway" or a

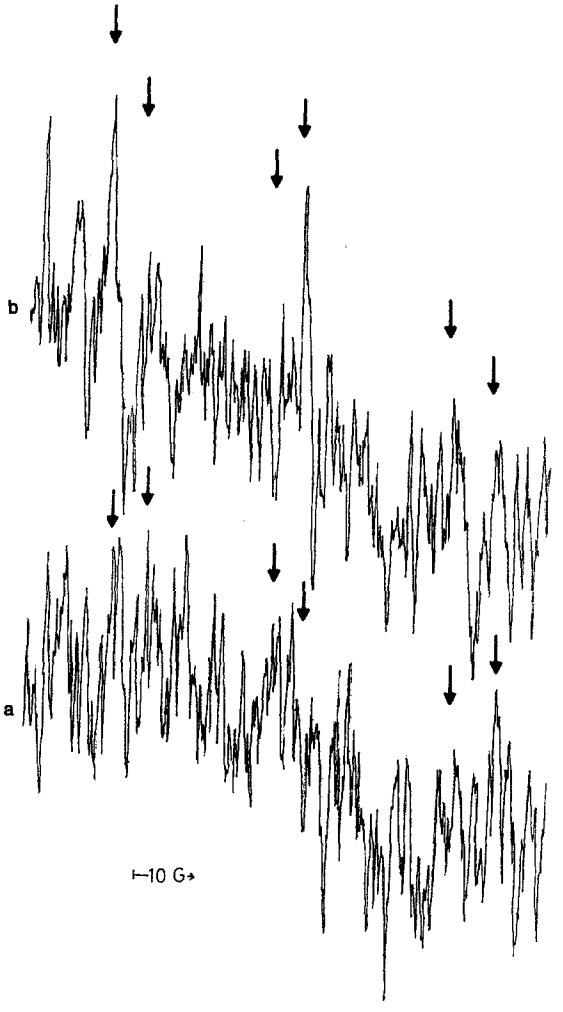

Figure 1. EPR spectra of $O_2^{\cdot -}$-PBN adduct formed in suspensions of leydig cells from [a] immature and [b] adult Mus musculus. Arrows represent the characteristic EPR absorption peaks of $O_2^{\cdot -}$-PBN adduct.

" Δ^5-pathway", with the former predominating in rodents and the latter in primates. In addition to the classical concepts that the key enzymes participating in the Δ^4-pathway converting pregnenolone to progesterone are Δ^5-3β-hydroxysteroid dehydrogenase and Δ^5-Δ^4-isomerase, evidences are available, however, to ascertain the operation of a free-radical modulated regulation at this level involving the ascorbate-peroxidase system (8,9). This has necessitated our search into the leydig cell steroidogenesis with an aim to demonstrate an enzyme-mediated production of H_2O_2 in these cells that might determine the peroxidase action. The high levels of superoxide dismutase and superoxide anion radical in the pubertal leydig cells confirm our hypothesis. The absence of both

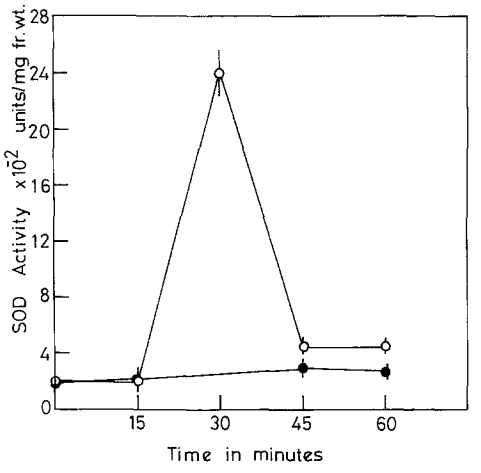

Figure 2. Effect of luteinizing hormone on the SOD activity associated with leydig cells in immature male Mus musculus[o]. Controls received injections of vehicle alone [●]. Assays were performed at various time intervals after LH-injection and the results presented are mean±s.e.m. of five replicates analyzed.

these factors in the leydig cells of pre-pubertal mice, and its induction by LH shows a direct modulation of the pregnenolone-> progesterone conversion by this pituitary hormone. The LH stimulated protein kinase of the leydig cells has been localized in the cytoplasm and depends upon cyclic AMP for its ability to phosphorylate proteins, mostly of that kind that regulate the early steps in steroidogenesis (14). In cell-free testicular homogenates, formation of labeled testosterone from cholesterol-4-^{14}C was found to be stimulated by ascorbic acid (15). Thus, it becomes evident that Δ^4 pathway of testosterone biosynthesis widely believed to proceed from pregnenolone en route pregnenedione to progesterone directed by Δ^5 3-β hydroxy steroid dehydrogenase and Δ^5-Δ^4 isomerase respectively can also proceed through an alternate pathway with a pregnenolone free radical as an intermediate. This alternate pathway seems to be mediated by superoxide dismutase and peroxidase with the former and presumably with the latter too, being induced in the leydig cells by luteinizing hormone. This identifies a new circuit in the regulatory switch that controls steroid biosynthesis in mammalian testis.

ACKNOWLEDGMENTS

The authors wish to acknowledge the financial support under DRS to School of Life Sciences, Devi Ahilya Vishwvidyalaya, Indore originated from University Grants Commission, New Delhi, India.

REFERENCES

1. Eik-Nes, K.B. (1975 a) Vitam. Horm. 33, 193-200.
2. Eik-Nes, K.B. (1975b) Handbook of Physiology (Grup, R.O. and and Astwood, E.B., eds.) Sect 7 : vol. 5 American Physiological Society, Washington DC, pp. 95-100.
3. Ewing, L. and Brown, B.L. (1977) In : The Testis (Johnson, A.J., Gomes, W.R. and Van Demark, N.L., eds.) vol. 4 Academic press, New York, San Fransisco, London, p. 239-250.
4. Torre, B de la, Hedman, M. and Diczfalusy, E. (1977) Acta Endocrinol. (Kbh.) 86, 851-860.
5. Moger, W.H. (1979) J. Endocrinol. 80, 321-326.
6. Betz, G., Tsai, P. and Hales, O. (1980) Endocrinology 107, 1055-1059.
7. Menard, R.H., Latif, S.A. and Purvis, J.L. (1975) Endocrinology 97, 1587-1590.
8. Agrawal, P. and Laloraya, M.M. (1977) Biochem. J. 166, 205-208.
9. Laloraya, M., Kumar, G.P. and Laloraya, M.M. (1988) Biochem. Biophys. Res. Commun. 157(1), 146-153.
10. Bellve, A. R., Millette, C.F., Bhatnagar, Y.M. and O' Brien, D.A. (1977). J. Histochem. Cytochem. 25, 480-494.
11. Schumacher, M., Schafer, G., Holstein, A.F. and Hilz, H. (1978) FEBS Letters, 91, 333-338.
12. Marklund, S. and Marklund, G. (1974) Eur. J. Biochem. 47, 469-474.
13. Frankenberger, W. and Blakemore, T. (1985) Program "Introductory Statistics Software Package ", Version 1.0, Addison-Wesley Publishing Co., USA.
14. Mann, T. and Lutwak-Mann, C. (1980) In: Male Reproduction Function and Semen. pp-106-110. Springer-Verlag, Berlin.
15. Carballeria, A., Fishman, L.M. and Durnhofer, F. (1974) Metabolism. 23(12), 1175-1184.