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CHANGES IN THE LEVELS OF SUPEROXIDE ANION RADICAL AND SUPEROXIDE DISMUTASE DURING THE ESTROUS CYCLE OF RATTUS NORVEGICUS AND INDUCTION OF SUPEROXIDE DISMUTASE IN RAT OVARY BY LUTROPIN

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Summary. Superoxide dismutase, which has been shown to be present in a number of tissues, exhibits cyclic changes during the reproductive cycle of rats. An inverse correlation is seen between the levels of superoxide dismutase and superoxide radical. In immature, pseudopregnant rats, primed with human Chorionic Gonadotropin, lutropin seemed to induce ovarian superoxide dismutase, which could be blocked significantly by the introduction of anti-LH serum. These results point out the specific induction of superoxide dismutase by lutropin. It is reasonable to postulate that during luteal functioning, luteinizing hormone induces superoxide dismutase which in turn seems to play a central role generating hydrogen peroxide from superoxide anion radicals. Hydrogen peroxide, thus formed, drives the peroxidase-ascorbate system, responsible for production of progesterone.

Introduction. Luteinizing hormone has been shown to induce peroxidase in the corpora lutea accompanying the depletion of ascorbate in the rat ovary, and suggestion has been made that the free radical of ascorbate produced by the action of peroxidase on ascorbic acid may trigger the oxidation of pregnenolone through a free radical mechanism, thus bringing about rapid formation of progesterone (1). For peroxidase to play such a pivotal role in luteal steroidogenesis, the generation of high levels of hydrogen peroxide - an essential substrate for all peroxidase mediated reactions, is a pre-requisite. Superoxide dismutase - the well known hemocuprein molecule - the blue copper protein isolated from bovine erythrocytes by Mann & Keilin in 1938 (2) has been assigned this enzymic function recently. It is an enzyme which catalyzes the

Abbreviations used:

SOD = superoxide dismutase; $O_2^{\cdot -}$ = superoxide anion radical; H_2O_2 = hydrogen peroxide; PMSG = pregnant mare serum gonadotropin; hCG = human chorionic gonadotropin; LH = luteinizing hormone (lutropin); PBN = N-t-butyl-α-phenyl nitrone; DDC = diethyl dithio carbamic acid, sodium salt; EDTA = ethylene diamine tetra -acetic acid; HEPES = N-2-hydroxy ethyl piperazine-N'-2-ethane sulfonic acid; HBSS = Hank's Balanced Salt Solution.

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dismutation or disproportionation of superoxide free radical anions to generate hydrogen peroxide (3). There have been earlier reports of the presence of this enzyme in the ovary but no functional correlation has been made as yet (4).

The toxicity of biological systems to superoxide anion radical is well documented. But the validity of this superoxide theory of oxygen toxicity has been challenged from studies upon anaerobes; it is being suggested that organisms living without oxygen would not make $O_2^{\cdot -}$ and thus would not need SOD. The demonstration of SOD in some anaerobes by Prof. Morris's laboratory in Wales might be taken to suggest that its true biological function is not that of removing oxygen (5). Till now, there has been no report of a useful role of $O_2^{\cdot -}$ in biological systems. For superoxide dismutase to play any role in luteal steroidogenesis, the requirement of this radical would be a must, since it is the substrate for this reaction.

Thus, in this paper we report changes in ovarian SOD and superoxide anion radical during the estrous cycle of albino rat. We also elucidate the induction of SOD by lutropin and the complete blockade of this induction by anti-LH serum to illustrate the specific nature of this LH effect.

Materials and Methods

Reagents. Pregnant Mare Serum Gonadotropin, human Chorionic Gonadotropin, Luteinizing hormone, Trizma base, Trizma HCl, Triton X-100, Diethyl dithio carbamic acid, sodium salt and N-2-Hydroxy ethyl piperazine- N'-2-ethane sulfonic acid were obtained from Sigma Chemical Company Inc., USA. N-t-butyl-α-phenyl nitrone was obtained from Aldrich, Milwaukee, WI. Pyrogallol was from Loba Chemie, India and Ethylene Diamine Tetra acetic acid from BDH, India. Anti-ovine LH was a gift from Prof. N.R.Moudgal, Indian Institute of Science, Bangalore, India. Tris-HCl buffer (50 mM, pH 8.2) was made by mixing 50mM Trizma base and 50mM Trizma HCl in the ratio of 2:1. 1mM EDTA was added to this solution and the pH adjusted to 8.2 at 27°C.

Animals. Female albino rats (Wistar strain) bred in our institute colony and housed in temperature (27±1°C) and light (14hr light : 10hr dark) controlled rooms were used for this study.

Method. Experiments during the estrous cycle were conducted on mature female rats of the age group, 4-6 months. The reproductive stage was identified by examination of vaginal smears (6). Only those female rats which showed a regular 4 or 5 day estrous cycle were used for the above study.

For experiments with lutropin, immature 40-50days old female rats were primed by an intra-peritoneal injection of 50IU PMSG followed by 25IU hCG delivered intraperitoneally, 48 hrs., later. The primed rats were used for experiments after 48 hrs., following the last injection. Two sets of experiments were carried out. The first concerned the effect of LH on SOD in immature females (Group I). For this, purified 20μg lutropin dissolved in 1ml of 0.9% sodium chloride, was administered to the rats by a subcutaneous injection as a single dose. The second set of experiment determined the changes in SOD in the LH injected hCG primed pseudopregnant rats after anti-LH treatment (Group II). A single subcutaneous injection of 20μg LH/ml was given to this set of animals also. Subsequently, circulating LH was neutralized with 40μg of anti-ovine LH serum dissolved in 1ml of 0.9% sodium chloride, delivered subcutaneously, 10minutes later as a single dose.

The rats were killed by cervical dislocation at each stage of reproductive cycle, viz., diestrous I, diestrous II, proestrous and estrous; and at different time periods of LH injection, viz., 15, 30, 60, 120 & 240

minutes. The ovaries were dissected, freed of adhering fat and blood was removed by washing several times with chilled physiological saline and were then used for analysis. The tissues were first weighed before processing for superoxide radical quantitation or superoxide dismutase assay. In all the experiments, 3 replicates were taken for each analysis.

Spin-trapping of superoxide radical generated by ovaries. Preweighed ovaries were homogenized in HBSS (7) adjusted to pH-7.2 using 50mM HEPES, at 2,500rpm (2cycles, 10seconds each) using a Polytron homogenizer with PT 10 accessory, so as to disaggregate the cells. The extract was incubated with 50mM PBN and $1x10^{-3}$M DDC (final concentration) for 1 hr., at 27°C. After incubation, 25µl aliquots were transferred to glass capillaries (Clinicon International, GmbH) and one end flame-sealed taking care not to warm the suspensions.

The radical was detected by incorporating the inhibitor of SOD viz., DDC, in the isolation medium and trapping the radical as a PBN adduct. EPR spectra of the PBN-superoxide radical adduct were recorded on a Varian E-104 EPR Spectrometer equipped with TM 110 cavity. Instrument settings employed were : Scan range- 100G, Field set- 3235G, Temperature- 27°C, Time constant- 0.5sec., Scan time- 8min., Modulation Amplitude- 2G, Modulation frequency- 100kHz, Microwave power- 5mW, Microwave frequency- 9.01GHz, Reciever gain- $2.5x10^{4}$ x10 (unless otherwise stated). The EPR absorption line intensities of the mid-field lines were calculated employing the equation $I=kW^{2}h$ (where $k=6.51x10^{-10}$, a line shape constant; W=line width; h=line height; I-is the integrated intensity of the first derivative signal) (8), which served as a measure to compare the quantity of superoxide radical generated.

Assay of superoxide dismutase activity in ovary of rats. The tissue was placed in 4ml chilled Tris-HCl buffer (50mM, pH-8.2) containing 1mM EDTA and homogenized at 4°C at a speed of 13,000rpm (2 cycles, 30 seconds each) using a Polytron homogenizer with PT 10 accessory. The homogenates were treated with 1ml of 1% Triton X-100 so that the final concentration of the detergent was 0.2%. After treatment for 20 minutes, the suspensions were centrifuged at a speed of 15,000rpm at 4°C using a Sorvall OTD 65B ultracentrifuge and a T865.1 fixed angle rotor. The pellets were discarded while the supernatants were assayed for SOD activity by the method of Marklund & Marklund, 1974 (9) using the ability of the enzyme to inhibit autoxidation of pyrogallol. The enzyme kinetics was monitered on a LKB Ultrospec 4050 Spectrophotometer equipped with peripheral Apple 2e PC and Epson FX 800 printer using the software - Program Enzyme Kinetics (LKB Biochrom Inc., Cambridge). All calculations were made as per milligram fresh weight.

Data Analysis. Statistical analysis were made using Introductory Statistics Software Package (ISSP), Version 1.0 (10). The degree of variance of the observations obtained was tested by subjecting them to a one-way ANOVA, where the results of each group is compared with that of the preceeding group. The Pearson Correlation Coefficient 'r' was calculated between the levels of SOD and $O_2^{\cdot-}$ using the above mentioned program.

Results and Discussion. Figure 1 (a, b, c & d) shows the EPR spectra of PBN-superoxide adducts when the $O_2^{\cdot-}$ radical generated from ovaries of rats at diestrous I, diestrous II, proestrous and estrous were trapped using 50mM PBN in the presence of an inhibitor of SOD viz., DDC ($1x10^{-3}$ M). Arrows represent the absorption line characteristics of PBN-superoxide adduct. The presence of $O_2^{\cdot-}$ in the system was confirmed by comparing the absorption line characteristics of PBN-superoxide adduct obtained when $O_2^{\cdot-}$ was generated artificially using a pyrogallol autoxidation system (Fig. 1, e). The PBN-superoxide adduct obtained is further verified by its characteristic nitrogen hyperfine splitting of a^{N} = 14.81G (11). The intensities of the mid-field line (indicated by solid arrows) were calculated and served as a

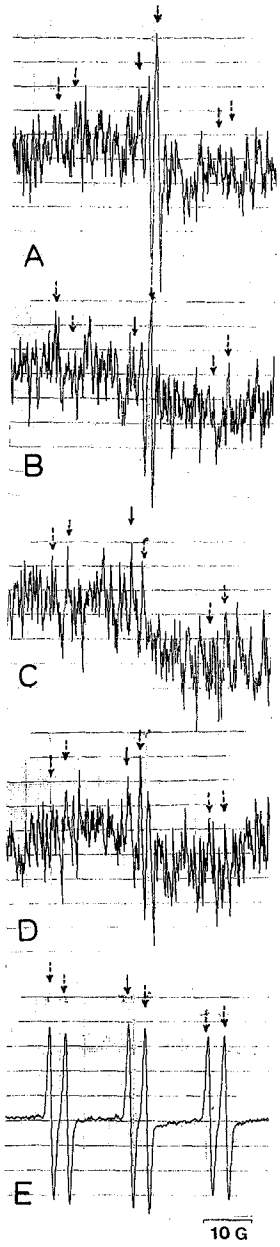

Fig. 1. EPR spectra of $O_2^{\cdot -}$-PBN adduct. Figures a, b, c & d represent EPR spectra obtained when ovaries of rats at diestrous I, diestrous II, proestrous and estrous were incubated with 50mM PBN [Ref., Materials and Methods]. Reference spectrum of $O_2^{\cdot -}$-PBN adduct [Fig. e] was recorded using a pyrogallol-autoxidation system. Instrument settings were : Field set- 3235G, Scan range- 100G, Modulation amplitude- 2G, Modulation frequency- 100kHz, Reciever gain- 2.5 x 10^4 x 10, Time constant- 0.5 sec., Microwave power- 5mW, Microwave frequency- 9.01GHz, Scan time- 8minutes, Temperature- 27°C. Arrows represent absorption line characteristics of $O_2^{\cdot -}$-PBN adduct. Intensities of mid-field absorption lines [represented by solid arrows] served as a comparative measure for the quantity of adduct formed.

semiquantitative measure of the amount of $O_2^{\cdot -}$ present in a given system. We could observe marked changes in the levels of $O_2^{\cdot -}$ radical during the reproductive cycle of rat. The ovary of rat at diestrous I exhibited maximum $O_2^{\cdot -}$ radical (Fig. 2). At diestrous II there was a highly significant ($P<0.01$) decline in the levels of $O_2^{\cdot -}$ which further decreased to very low quantities ($P<0.01$) during proestrous. A build up in the levels of this radical ($P<0.01$) was observed at estrous.

Figure 2 also exhibits the changes in SOD activity during the different stages of estrous cycle of rat. There is no significant difference in the levels of SOD during diestrous I and diestrous II. A sharp rise is seen in the amount of SOD at proestrous, the difference between the levels during diestrous II & proestrous being highly significant ($P<0.01$). There is a marked

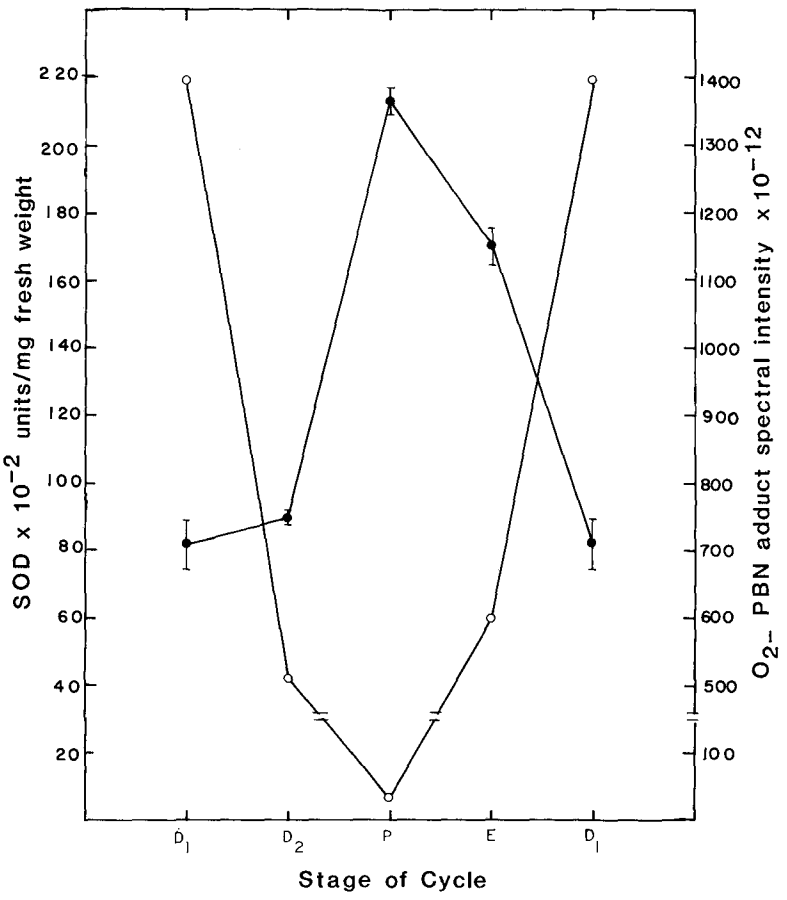

Fig. 2. Changes in the levels of superoxide anion radical [o] and superoxide dismutase [•] during the course of the reproductive cycle of albino rats. Data represented are mean values ± standard error of the mean [s.e.m.]. D_1- diestrous I, D_2- diestrous II, P- proestrous and E- estrous. Experimental details are given in Materials and Methods section.

decrease (P<0.01) in SOD levels during estrous which further continues to fall during diestrous I.

The changes in SOD activity after lutropin treatment are represented in figure 3. The SOD activity rises within 15 minutes of LH injection and reaches a peak (P<0.01) in 30 minutes of lutropin treatment. A sharp decline is seen in the levels of SOD after 60 minutes of injection (P<0.01). Further, at 2hr., and 4hr., of LH administration there is no significant change in the levels of SOD from those at 1hr. Figure 3 also shows the effect of anti-LH serum on LH induced changes in SOD activity with time after anti-LH treatment in pseudopregnant rats. There is no significant change in the levels of SOD with time after anti-LH treatment, which continue to remain at their basal level. The superoxide dismutase activity at 15 and 30 minutes after LH treatment was

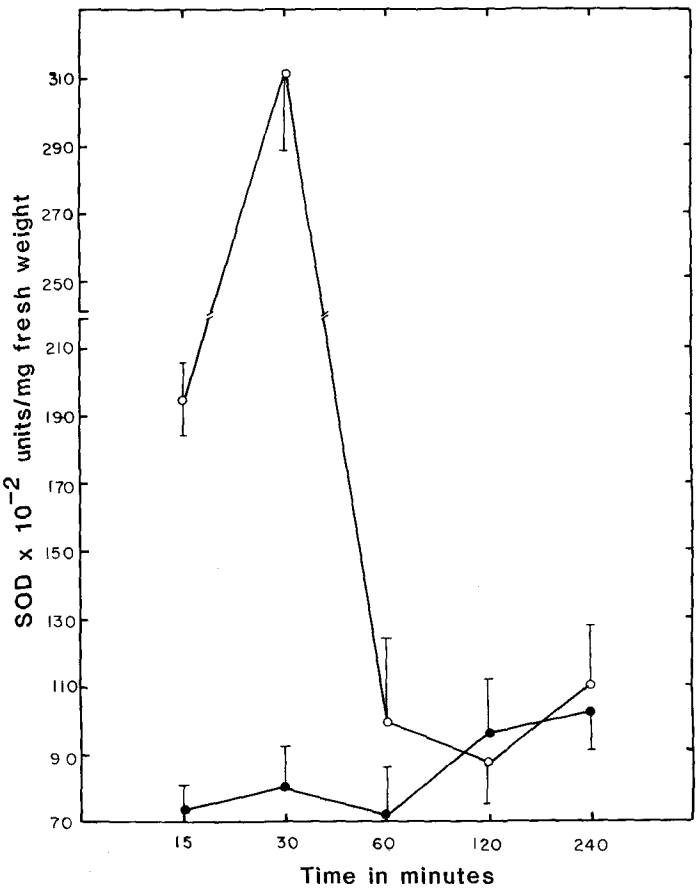

Fig. 3. Lutropin-induced changes in superoxide dismutase in immature pseudopregnant rats and the effect of anti-LH serum on the above changes. o——o Change in SOD activity after LH administration. Rats were killed at 15, 30, 60, 120 and 240 minutes after LH administration. ●——● SOD levels in anti-LH injected rats. Values indicated are mean ± s.e.m. Experimental details are given in Materials and Methods section.

significantly different ($P<0.01$) from the corresponding levels noticed after anti-LH administration. After 30minutes, i.e., at 60, 120 & 240 minutes the levels of SOD in both the groups of animals did not show statistically significant differences (fig. 3).

Thus, our results presented above (fig. 1 & 2) clearly show the first mentioned presence of superoxide free radical in ovarian tissue and its cyclic changes during the course of reproductive cycle. An inverse correlation between SOD and $O_2^{\cdot-}$ radical levels is clearly evident ($r=-0.77$) (fig. 2). Our results on SOD corroborate very well with those on peroxidase (1) since the observed high levels of SOD during proestrous would generate sufficient hydrogen peroxide to make the substrate available for peroxidase action at estrous, the levels of which reach a peak during estrous to bring about luteal steroidogenesis. Our work on pseudopregnant rats also show the induction of SOD by LH treatment and also points to a well-timed relationship since our results demonstrate a peak in SOD, 30minutes after LH administration (fig. 3) which correlates very well with a peak of peroxidase between 2 & 3 hr., after lutropin treatment (1) ensuring sufficient levels of H_2O_2. The complete inhibition of SOD induction by anti-LH serum in hCG-primed pseudopregnant rats, strengthens our idea of the induction of SOD by LH thereby confirming the specificity of LH action.

Uptill now, there has been no report on the presence of $O_2^{\cdot-}$ radical in the ovarian tissue, a radical known for its toxic effects (12). In this paper, we demonstrate the presence of this radical and we suggest a beneficial role for this radical in regulating luteal steroidogenesis by a peroxidase dependant free radical reaction involving ascorbate for the oxidation of pregnenolone to progesterone. We also suggest that during lutropin action, in the sequential events leading to luteal steroidogenesis, the first step, when LH acts, may be the induction of SOD (fig. 2 & 3) which leads to the production of H_2O_2 for the peroxidase to be functional. Monoamine oxidase is another enzyme which is reported to show cyclic changes during reproductive cycle and could be another source to generate H_2O_2 for the system (13). It appears that lutropin may be regulating luteal steroidogenesis by inducing a multicomponent oxidase-peroxidase system with SOD as one of its components. We conclude that SOD is generated at a time prior to peroxidase induction to produce enough H_2O_2 for a well timed sequential action.

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