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DISCOVERY OF A NOVEL AND ALTERNATE HYDROGEN PEROXIDE GENERATION MACHINERY IN THE MAMMALIAN THYROID GLAND THAT MODULATES IODINATION OF TYROSINE

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The hydrogen peroxide-dependent and peroxidase-mediated iodination of tyrosine, a pivotal step in the thyroxine biosynthesis in thyroid glands of mammals, has always been thought to be dependent on a glucose-glucose oxidase system to meet its demand for hydrogen peroxide. This report documents a novel and probably kinetically faster hydrogen peroxide-generating system operating in the thyroid gland cells involving the superoxide dismutase and superoxide anion radical. In the female cycling mice, the superoxide radical and superoxide dismutase activity are shown to be at persistently high levels, quite likely acting as a potent hydrogen peroxide synthesizer in the mammalian thyroid gland. The significance of this discovery in relation to the thyroid physiology is discussed.

Thyroid peroxidase, a hemoprotein, is a membrane-bound enzyme that catalyzes the biosynthesis of thyroid hormone (1,2). The essential role of this enzyme is believed to be the incorporation of iodine into the tyrosyl residues of thyroglobulin (2). Iodide ion is presumably oxidised before being incorporated in tyrosine. The mechanism of peroxidation and then organification of iodide into thyroglobulin is thoroughly studied and is found to be thyroid peroxidase mediated in the presence of high concentrations of hydrogen peroxide, its substrate (3).

Abbreviations

SOD = superoxide dismutase; $O_2^{\cdot -}$ = superoxide anion radical; H_2O_2 = hydrogen peroxide; PBN = N-t-butyl-α-phenyl nitrone; DDC = diethyl dithio carbamic acid, sodium salt; DTPA = diethylene triamine pentaacetic acid; HBSS = Hank's Balanced Salt Solution.

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For thyroid peroxidase to play such an important role in thyroxine biosynthesis, generation of H_2O_2 in high amounts is a prerequisite. Although it has been assumed by various workers that glucose-glucose oxidase system is responsible for hydrogen peroxide generation (4), available data do not seem sufficient to prove its role. Few workers also mention presence of xanthine oxidase in thyroid for this function.

Superoxide dismutase is one of the major biochemical discoveries of the last twenty years and much attention has been directed towards the ubiquitous presence of SOD and free radicals. Superoxide dismutase, a haemcuprein having a molecular weight 31,000 D, discovered by Mann & Keilin,has been assigned the role of dismutating superoxide radical (5). The toxicity of this radical has been confirmed at many levels of normal and pathological cellular structure and function, including cell death, membrane peroxidation, DNA damage and loss of enzyme activity. But, involvement of $O_2^{\cdot -}$ and SOD in the biosynthesis of hormones like progesterone has been postulated recently by Laloraya et al (6,7,8,) in ovary and uterus.

The presence of high levels of peroxidase in the thyroid gland and the established involvement of the H_2O_2- peroxidase system in the iodination of tyrosine has invoked the idea of the participation of alternate H_2O_2 generating pathways, one of which being the superoxide dismutase that generates H_2O_2, utilizing a toxic oxygen free radical viz., $O_2^{\cdot -}$ (9). Thus, this study has been an attempt to assay the SOD activity of thyroid homogenates and the superoxide radical generating capability of thyroid follicular suspensions during the various stages of the reproductive cycle of mature female albino mice. The functional correlations have also been drawn from the observations reported in this article.

MATERIALS AND METHODS

Reagents. Trizma base, Trizma HCl, Triton X-100, Diethyl dithio carbamic acid and Diethylene triamine pentaacetic acid were purchased from Sigma Chemical Company Inc., USA, while N-t-butyl-α-phenyl nitrone was from Aldrich, Milwaukee, WI. Pyrogallol was of Loba Chemie, India and all other chemicals were of reagent grade. Tris-HCl (pH 8.2) was prepared as described elsewhere (6).

Animals. Female Mus musculus (swiss strain) were bred in our institute colony and housed in temperature ($27\pm1^{\circ}C$) and light (14h light : 10h dark) controlled rooms. The females showing normal 4 or 5 day estrous cycle were used for study.

Method. The reproductive stage of the animal was checked by examination of vaginal smears of sexually mature virgin female mice of the age group 3-4 months (10). The females were killed by cervical dislocation at different stages of estrous cycle. The thyroid gland was taken out, cleared of adhering fat and washed several times with chilled physiological saline (0.154N NaCl) to remove blood. The tissue was weighed and then processed for SOD assay and superoxide radical quantitation. In all cases 3 replicates were taken for each analysis.

Superoxide dismutase activity assay in thyroid gland. Preweighed thyroid tissue was taken in 4ml of chilled Tris-HCl buffer of molarity 50mM (pH 8.2) containing 1mM DTPA and then homogenized in a Polytron homogenizer with PT 10 accessory. The homogenate were treated with Triton X-100 (1%) v/v for 30 minutes and then the suspension was centrifuged for 30 minutes at a speed of 15,000 rpm at $4^{\circ}C$ in a Sorvall OTD 65B Ultracentrifuge. The pellet was discarded and the supernatant was subjected to SOD activity assay by the method of Marklund and Marklund (11) which involves the ability of enzyme to inhibit autoxidation of pyrogallol. The enzyme kinetics was carried on a LKB Ultrospec 4050 spectrophotometer attached with peripheral Apple 2e PC and Epson FX 800 printer. All calculations were made as per milligram fresh weight.

Protocol for spin-trapping of superoxide radical generated in thyroid gland. $O_2^{\cdot -}$ was trapped as a PBN-adduct essentially in the same manner as described elsewhere (6). After dispersing the follicle cells in 1ml HBSS (pH 7.2) (12), the extract was incubated with 50mM PBN and 1×10^{-3}mM DDC (final concentration) for 1hr at $27^{\circ}C$. After incubation, 50ul aliquots were taken in a glass capillary (Clinicon International, GmbH) and one end flame sealed taking care not to warm the suspension. DDC was used as a SOD inhibitor. EPR spectra of PBN-radical adduct were recorded on a Varian E-104 EPR spectrometer equipped with TM 110 cavity. Instrument settings were : Scan range- 100G, Feild set- 3237G, Temperature- $27^{\circ}C$, Time constant- 0.5sec., Scan time- 8min., Modulation amplitude- 2G, Modulation frequency- 100kHz, Microwave power- 5mW, Microwave frequency- 9.01GHz and Receiver gain- $2.5 \times 10^4 \times 10$. The EPR absorption line intensities of the low, mid and high-field lines were calculated employing the equation $I = kW^2h$ (where $k = 6.51 \times 10^{-10}$, a line shape constant ; W= line width; h= line height; I- is the integrated intensity of the first derivative signal) (13), and the average of them served as a measure to compare the quantity of superoxide radical generated.

Statistical Analysis. Statistical analysis was conducted utilising Introductory Statistics Software Package (ISSP), Version 1.0 (14). The degree of variance of the results of each group was compared with that of the preceeding group by subjecting them to a one-way ANOVA. The Pearson correlation

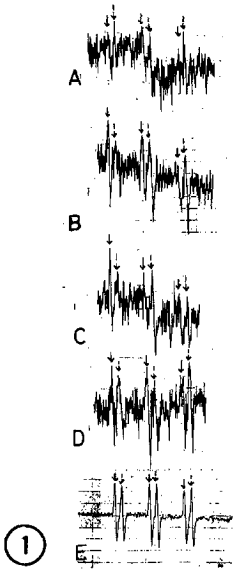
coefficient 'r' between the levels of superoxide anion radical and SOD was calculated using the above mentioned program.

RESULTS AND DISCUSSION

Figure 1 [A, B, C and D] present the spectra of PBN-superoxide radical adducts from thyroid at diestrous I, diestrous II, proestrous and estrous. The superoxide radical was trapped by PBN in presence of an inhibitor of SOD, i.e., DDC. The PBN free-radical adduct spectra obtained were compared with a standard PBN-$O_2^{\cdot -}$ adduct spectra recorded using a pyrogallol autoxidation system [Figure 1, E]. The similarities in the EPR spectra and characterstic nitrogen hyperfine splitting of a^N= 14.81G have been carefully analysed to identify the PBN-$O_2^{\cdot -}$ adduct formed. The intensities of low, mid and high-field absorption lines were averaged and we observed an almost equal level of $O_2^{\cdot -}$ during diestrous II and proestrous, which showed a significant fall during estrous stage ($p< 0.01$).

Figure 2 shows the level of SOD in thyroid during different stages of estrous cycle. The levels of SOD in thyroid gland seems to be at persistently high levels during different stages of estrous cycle when compared to what was reported earlier from our laboratory during the investigations on yet another tissue, viz., ovary (6). A positive correlation between SOD and superoxide radical levels is clearly evident ($r= 0.97$) in figure 2. Our results showing constant high levels of SOD during estrous cycle correlates well with constant high levels of $O_2^{\cdot -}$ thus ensuring sufficient production of H_2O_2; which is a prerequisite for iodine incorporation. Both SOD and superoxide radical show a peak during proestrous.

The system responsible for H_2O_2 generation has been a matter of considerable debate. Few workers have shown doubtful results



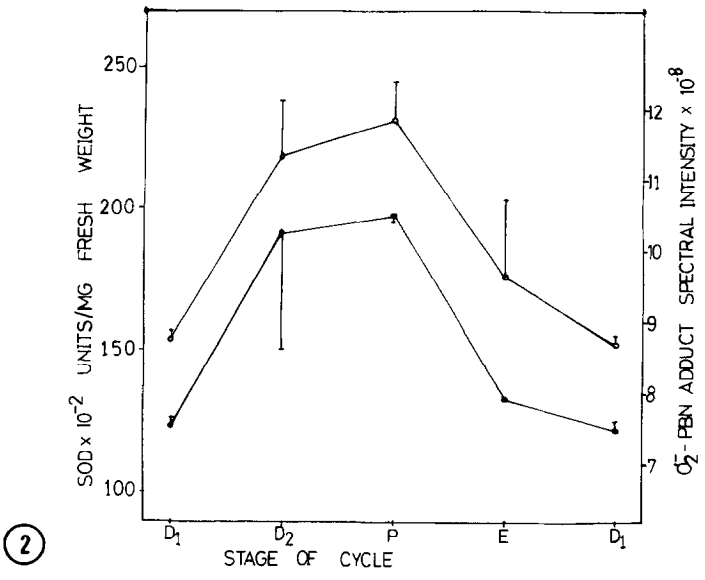


Figure 1. EPR spectra of $O_2^{\cdot -}$-PBN adduct. Figures A, B, C and D represent EPR spectra obtained when thyroid gland of mice at diestrous I, diestrous II, proestrous and estrous were incubated with 50mM PBN [Ref., Materials and Methods]. Reference spectrum of $O_2^{\cdot -}$-PBN adduct [Figure E] was recorded using a pyrogallol autoxidation system. Instrument settings were : Field set- 3237G, 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- 8min., Temperature- 27°C. Arrows represent absorption line characterstics of $O_2^{\cdot -}$-PBN adduct. Intensities of low, mid and high-field [represented by solid arrows] were averaged and served as comparative measure for quantity of adduct formed.

Figure 2. Changes in the levels of superoxide anion radical [•] and superoxide dismutase [o] during the course of reproductive cycle of albino mice. 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.

about the direct involvement of xanthine oxidase and glucose-glucose oxidase system (4) in H_2O_2 generation.

In 1953, Weiss (15) reported cupric ion supplemented iodination of tyrosine. Hati and Dutta (16) could later isolate a microsomal enzyme preparation from goat submaxillary gland in which cupric ion was needed by the enzyme for its activity to promote iodination. Cunningham and Kirkwood (17) also reported iodination of tyrosine to monoiodotyrosine by partially purified copper dependent enzyme from rat submaxillary gland. In 1969, Hati and Dutta (18) suggested the presence of at least two

separate enzymes: (a) one catalysing production of H_2O_2, which required presence of cupric ion (b) the other enzyme catalyzing the iodination of tyrosine, which is probably a peroxidase that requires H_2O_2.

The constantly high levels of SOD activity in the mouse thyroid gland reported here clearly demonstrate that the copper containing enzyme that is assigned the role in iodination of tyrosine is superoxide dismutase. It seems that the thyroid gland has evolved a very efficient mechanism that transfigures a whole sequence of free radical chain reaction that would have taken place into an H_2O_2-dependent and peroxidase-mediated tyrosine iodination which is of vital importance as far as the thyroid functioning is concerned. Another interesting feature of the tyrosine-iodinating machinery is that reducing agents like glutathione reduced and cysteine inhibited iodination (19).

Recent observations from our laboratory (20) show that reduced thiol compounds including cysteine and glutathione strongly inactivate SOD, in the light of which Taurog's report (19) can be well interpreted that the reducing compounds inhibited iodination of tyrosine through the inactivation of SOD, thus giving added weight to the fact that SOD is the major H_2O_2 generating enzyme that drives the tyrosine iodination. Further, this H_2O_2-generating machinery is cycle-independent in the female mice, meeting the requirements of the thyroid gland. A slightly increased level of SOD and superoxide radical is seen during proestrous and the reason for this cannot be pinpointed to a specific function but the increase may probably result in increase in thyroxine hormone production via increase in H_2O_2 mediated iodination. This thyroxine hormone would increase the basal metabolic rate which is quite natural since there is increase in some specific hormone biosynthesis in females during

proestrous like progesterone. Thus, we demonstrate that superoxide dismutase working in a tandem with peroxidase is involved in the iodination of tyrosine, which is the pivotal step in the biochemical sequence of thyroxine production. Further studies are in progress to elucidate the exact role of superoxide radical and SOD in tyrosine iodination.

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