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Antioxidant Enzyme Activity in the Maturing Rat Testis

VILLE PELTOLA, ILPO HUHTANIEMI, AND MARKKU AHOTUPA

From the Department of Physiology, University of Turku, Turku, Finland.

ABSTRACT: Developmental profiles of the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), glutathione transferase (GSH-Tr), and hexose monophosphate shunt (HMS) were measured in the rat testis and liver. The level of SOD in the testis was high at the age of 6 to 10 days, after which it dropped to approximately one third of that level by 20 days of age, and remained there up to 8 months of age. In the liver, SOD activity steadily increased from the neonatal to adult stage of life, reaching the same level as detected in the testis. The testicular activity of catalase was only 2% to 7% of that found in liver at all ages. It increased in both organs up to 6 weeks of age, whereafter the hepatic activity gradually decreased and no further changes were seen in the testis. The GSH-Px activity was low in the testis and declined slightly with age, whereas activity in the liver increased four-fold between birth and adulthood. The activity of GSH-Tr was similar in both organs studied: it increased after birth, showing a maximum in the liver at 1.5 months (ten-fold increase) and in the testis at 5 months of age (four-fold increase). The HMS activity was two to three times higher in the liver than in the testis, and decreased slightly with age in both organs. Thus, the basal levels and developmental profiles of antioxidant enzymes in the testis differ greatly from those in the liver. The very high ratio of SOD to catalase plus GSH-Px in testis (0.92 to 3.48), compared with that found in the liver (0.009 to 0.083), makes the former especially vulnerable to the harmful effects of reactive oxygen species. The peak in SOD activity at 6 to 10 days of age might be related to the physiologic events leading to cell differentiation at this time.

Key words: rat testis, reactive oxygen, superoxide dismutase, catalase, development, cell differentiation.

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Potentially toxic reactive oxygen species are produced in normal cellular metabolism and in abundance in prooxidant states. Reactive oxygen species are degraded by the organized system of antioxidant enzymes. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion radical ($O_2^{\cdot -}$) to hydrogen peroxide, which is further metabolized by catalase, and to a lesser extent by glutathione peroxidase (GSH-Px; Fridovich, 1983). Hydrogen peroxide is not a particularly reactive product, but it may be reduced to the highly reactive metabolites hydroxyl radical ($OH\cdot$) or singlet oxygen ($^1O_2$; Halliwell and Gutteridge, 1984). In prooxidant states, lipid peroxides are formed from polyunsaturated fatty acids of biomembranes, causing a chain reaction that leads to deterioration of the membrane structure and integrity (Slater et al, 1987). GSH-Px and glutathione transferase (GSH-Tr) function in the detoxification of reactive lipid peroxides (Chiu et al, 1982), and nicotinamide adenine dinucleotide phosphate (NADPH) formed in the hexose monophosphate shunt (HMS) is needed in the regeneration of GSH. Testicular membranes are rich in polyunsaturated fatty acids, and thus susceptible to peroxidation injury.

Several studies have suggested a causative role for lipid peroxidation in the etiology of male infertility due to defective sperm function (Jones et al, 1979; Alvarez et al, 1987; Aitken et al, 1989). In accordance, antioxidant enzyme activity has been shown to decrease in experimental cryptorchidism, resulting in increased lipid peroxidation (Ahotupa and Huhtaniemi, 1992). Increased lipid peroxidation in the testis after the administration of testicular toxins, such as ethanol (Rosenblum et al, 1985) or cadmium (Kojima et al, 1990), may also contribute to the suggested vulnerability of this organ to oxidative stress. On the other hand, appropriate production of reactive oxygen species has been suggested to play a physiologic role in sperm–zona interaction (Aitken et al, 1989). Moreover, there is evidence of a physiologic role of reactive oxygen species in cellular differentiation (see Sohal and Allen, 1986; Sohal et al, 1986). Thus, production of reactive oxygen species in testicular tissue and control of these phenomena may have important physiologic and toxicologic consequences. In the present study, we investigated the physiologic development of the enzymatic antioxidant system in the rat testis from the age of 6 days to adulthood. For comparison, the same measurements were also carried out in the rat liver.

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Correspondence to: Ville Peltola, Department of Physiology, University of Turku, Kiinamyllynkatu 10, SF-20520 Turku, Finland.

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Materials and Methods

Chemicals

Butylated hydroxyanisole, cumene hydroperoxide, epinephrine, glucose-6-phosphate, hydrogen peroxide (H_2O_2), reduced gluta-

thione (GSH), reduced NADPH, as well as the enzymes catalase (bovine liver), GSH reductase (Bakers Yeast) and SOD (Cu/Zn-form, bovine erythrocytes) were all purchased from the Sigma Chemical Company (St. Louis, MO).

Animals

Male Sprague-Dawley rats were fed standard laboratory rat chow and water ad libitum after weaning at 21 days of age. The animals were kept at 20°C to 22°C with a light:dark cycle of 14:10 hours. Testes were removed from rats killed at the ages of 6 days, 10 days, 20 days, 31 days, 46 days, 2 months, 3 months, 5 months, and 8 months. Five animals were included in each group, except for the groups killed at the ages of 6 days (n = 6) and 8 months (n = 4). Liver tissue was collected and analyzed from another group of animals. The rats in the first age group were fetuses of 19 to 20 days of gestation (E 19–20, n = 11). The ages of the rats in the other groups were 1 day (n = 11), 3 days (n = 12), 7 days (n = 6), 10 days (n = 6), 19 days (n = 5), 33 days (n = 10), 44 days (n = 5), 2 months (n = 4), 3 months (n = 4), 5 months (n = 4), and 8 months (n = 4). The testes of the 6-day-old group were pooled and two pools were made of the testes from the 10-day-old group. Livers of the fetuses and the 1-day-old, 3-day-old, 7-day-old, and 10-day-old rats were combined into two pools to get enough tissue for measurement. Animals were weighed and decapitated at 19 days or later after anesthetizing with 100% carbondioxide; younger animals were decapitated without anesthesia. Fetuses were collected after decapitation of anesthetized mothers. Exposure to carbondioxide was limited by killing the animals immediately after they reached unconsciousness so that it would not have any effect on enzyme activity.

The onset of puberty in the colony of rats used in the study has previously been reported to occur at 44 to 49 days of age (Kolho et al, 1988). The lifespan of Sprague-Dawley rats is approximately 20 to 27 months (Berg, 1960; Menich and Baron, 1984), thus our oldest age group (8 months) represents adult, but not aged rats.

Tissue Preparation

After removal, testes and livers were rinsed in 0.25 mol/l sucrose and weighed. A 33% (w/v) testis homogenate was prepared with Ultra-Turrax homogenizer (Janke, Kunkel KG, Staufen, Germany) and a 20% (w/v) liver homogenate with a Potter-Elvehjem glass-Teflon homogenizer, both in a 0.25 mol/l sucrose solution (at 0°C). A postmitochondrial supernatant was prepared by centrifugation (10,000 *g* for 10 minutes at 4°C).

Enzyme Assays

Superoxide dismutase (Cu/Zn-form; enzyme commission no. 1.15.1.1) was assayed spectrophotometrically by inhibition of epinephrine autoxidation (Misra and Fridovich, 1972). Samples were added to reaction mixture made in sodium carbonate buffer, pH 10.2, and the absorbance change was recorded at 480 nm. Activity of catalase (EC 1.11.1.6) was determined by measuring the rate of disappearance of 15 mmol/l hydrogen peroxide at 240 nm in phosphate buffer, pH 7.0 (Beers and Sizer, 1952). In the analysis of SOD activity, 1 μg of the purified enzyme preparation corresponds to 3.6 U, and 1 μg of catalase corresponds to 2.5 U. GSH-Px (EC 1.11.1.9) was assayed in Tris-EDTA buffer, pH 7.6, by measuring the oxidation of $NADPH^+$ at 340 nm, with cumene hydroperoxide as the substrate (Paglia and Valentine, 1967). Assay of GSH-Tr (EC 2.5.1.18) was performed in phosphate buffer, pH 6.5; 1-chloro-2,4-dinitrobenzene was used as the substrate and the absorbance change was recorded at 340 nm (Habig et al, 1974). Hexose monophosphate shunt activity was assayed in Tris-HCl buffer, pH 7.5, using glucose-6-phosphate as the substrate and 340 nm as the recording wavelength (Glock and McLean, 1953). The situation of the measured values on the linear change of activity on the standard curve was controlled. SOD and catalase activities were determined in tissue homogenates, whereas centrifuged supernatant fluid was used in GSH-Px, GSH-Tr, and HMS assays.

Protein

Protein content was measured by the biuret method (Layne, 1957) with bovine serum albumin as the reference protein.

Statistical Analysis

One-way analysis of variance followed by Duncan's multiple-range test was used to compare the groups of rats 19 days or older to the rats in the 3-month-old age group, which was regarded as the control group of adult age. At ages of younger than 19 days, normal statistical analysis could not be performed, since the individual tissue samples were pooled for the measurements. However, since there is no reason to suggest the standard deviation to be larger for the young groups, we performed the same analysis on these groups using simulated individual values, which gave the calculated mean and standard deviation for the groups in question. The standard deviation was estimated to be the same (as percent of the mean) as the largest measured standard deviation in the older age groups. The statistical differences found between these groups and the 3-month-old age group are referred to in the Results section, but are not shown in the figures.

Results

Significant differences in the enzyme levels of the groups between 19 days and 8 months of age compared with those of the 3-month-old group are shown in the figures. The activity of all the enzymes measured in the testis of the groups between 6 and 10 days of age and in the liver of the fetuses and the groups 10 days of age or younger differed significantly ($P < 0.01$; see Materials and Methods) from the levels in the 3-month-old rats, with the exception of catalase and HMS at 10 days of age in the testis and HMS at 1 day, 3 days, and 10 days of age in the liver.

Superoxide Dismutase

The activity of SOD in the testis was very high in the youngest age groups (6 and 10 days of age; Fig. 1). Thereafter, it decreased to one third of that level by 20 days of age, and remained unchanged thereafter. In the liver, the development of SOD activity was different, increasing approximately eight-fold from the age of 3 days to the age of 3 months. A slight drop occurred after 3 months of age.

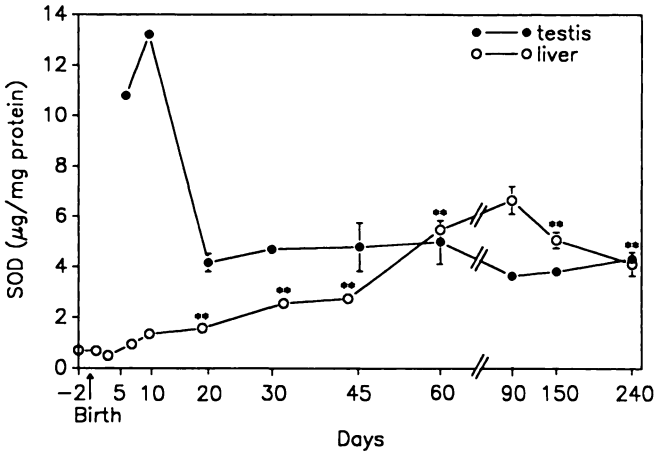

FIG. 1. Superoxide dismutase (SOD) activity in the maturing rat testis and liver. The 6-day-old group represents the mean of a pool of 6 pairs of testes, and the 10-day-old testis group and the fetal and 10-day-old or younger liver groups represent means of two pools of organs from 5 to 12 animals. The other age points represent the mean of 4 to 5 animals. At 19 days of age or older, SEM are represented by bars. The invisible error bars beyond the age of 19 days are within the symbols. The asterisks indicate a significant difference between the levels of the groups between 19 days and 8 months of age and the levels of the 3-month-old group: *, $P < 0.05$; **, $P < 0.01$. See Materials and Methods and Results for the statistical analysis of the younger age groups.

Catalase

The activity of catalase increased during the period of rapid growth in the testis and liver (Fig. 2). The testicular level was very low (2% to 7%) compared with the level in the liver. Catalase activity remained constant in the testis in adult rats but decreased in the liver with advancing age.

Glutathione Peroxidase

Testicular GSH-Px activity decreased until the age of 20 days, and thereafter increased only slightly with age (Fig. 3). In the liver, the activity of GSH-Px increased from the age of 3 days onward. With the exception of the youngest age groups (6 and 10 days of age), GSH-Px activity in testis was only 5% to 7% of that measured in the liver.

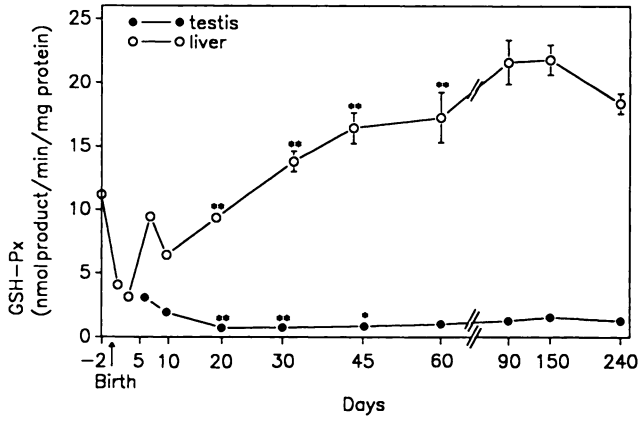

FIG. 3. Glutathione peroxidase activity in the maturing rat testis and liver. See Figure 1 for details.

Glutathione Transferase

Activity of GSH-Tr increased in the testis from 10 days of age onward, reaching its highest level at 5 months of age (Fig. 4). In the liver, GSH-Tr activity was lowest in the first days after birth, but increased steadily until the age of 1.5 months. After this age, there was a clear decline in hepatic GSH-Tr activity.

Hexose Monophosphate Shunt

The dependence of HMS on age is shown in Figure 5. Marked fluctuation of HMS activity was seen in the liver, particularly during the first 20 days of life. In the testis, the developmental profile of HMS was steadier, and the level of HMS activity was generally lower than in the liver.

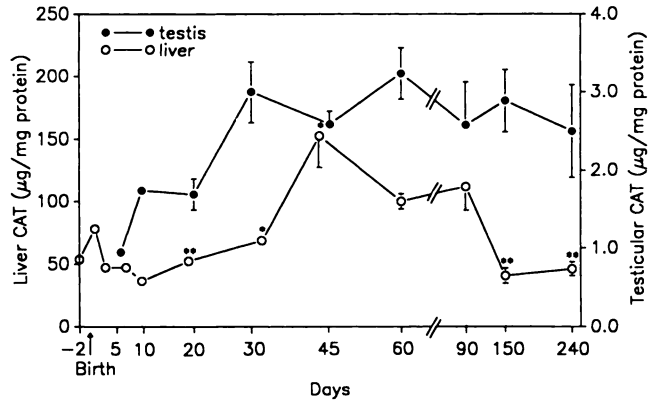

FIG. 2. Catalase activity in the maturing rat testis and liver. See Figure 1 for details.

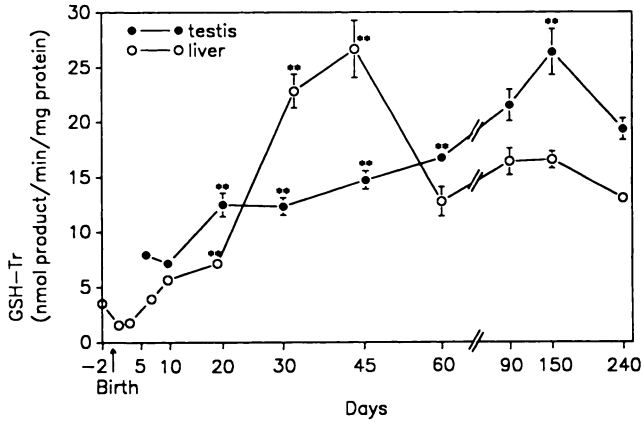

FIG. 4. Glutathione transferase activity in the maturing rat testis and liver. See Figure 1 for details.

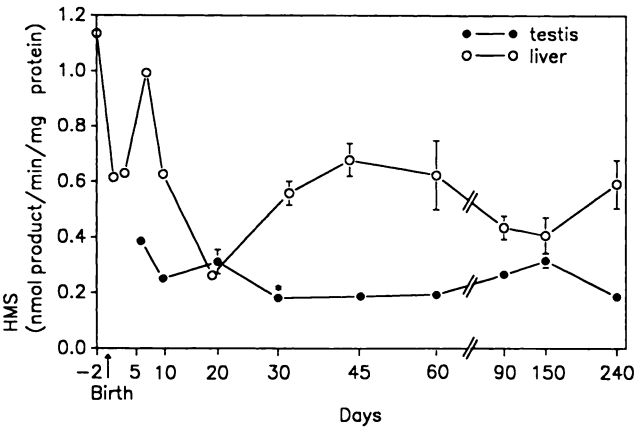

FIG. 5. Hexose monophosphate shunt activity in the maturing rat testis and liver. See Figure 1 for details.

Ratio of Superoxide Dismutase Activity to Catalase Plus Glutathione Peroxidase Activity

The ratio of SOD activity to the combination of catalase and GSH-Px activities was conspicuously high in the testis, being 11 to 150 times higher than in the liver (Fig. 6). In the testis, this ratio was highest in the young age groups (6 to 10 days of age), whereas in the liver it increased five-fold between birth and adulthood.

Discussion

The testicular antioxidant enzyme system was found to be different from that of the liver in several ways. The clearest differences were the high activity of SOD at 6 to 10 days of age and the conspicuously low activity of catalase and GSH-Px in the testis. Antioxidant enzymes constitute a system in which the right proportions of different enzymes are

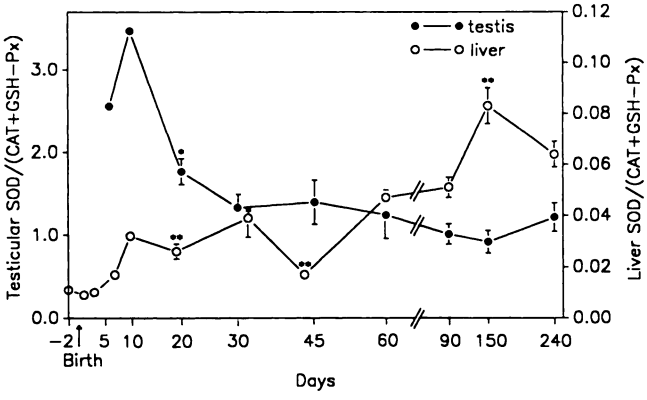

FIG. 6. The ratio of superoxide dismutase activity to the combination of catalase and glutathione peroxidase activity in the maturing rat testis and liver. See Figure 1 for details.

needed. The disproportionately high level of SOD activity in mouse cells transfected with the gene for human SOD has been found to lead to increased lipid peroxidation (Elroy-Stein et al, 1986). The high ratio of SOD activity to combined catalase and GSH-Px activity in testis suggests that it is vulnerable to prooxidative factors. However, nonenzymatic antioxidants, which were not examined, form an essential part of the defense system against prooxidative factors (Cadenas, 1985).

The greatest changes in testicular antioxidant enzyme activities occurred before the age of 20 to 30 days, whereafter the developmental profiles of all enzymes studied were steadier. Previously reported age-dependent changes in the testicular activity of GSH-Px (Behne et al, 1986) and GSH-Tr (Mukhtar et al, 1978) in the rat are in accordance with the present data. A similar low testicular activity but a difference in the developmental profile of catalase has been reported in the rabbit (Ihrig et al, 1974).

A possible explanation for the changes in the young age groups could be concomitant changes in testicular structure and function. Fetal-type Leydig cells disappear soon after birth and are replaced by adult-type Leydig cells (Roosen-Runge and Anderson, 1959; Tapanainen et al, 1984). The latter cells increase in number during puberty, but the volume of Leydig cells in the rat remains small (2% to 3%; Mori and Christensen, 1980). The proliferation of Sertoli cells is at its maximum just before birth, and ceases by the age of 3 weeks (Orth, 1982). Testicular growth is mainly due to an increase in germ cell number. The onset of spermatogenesis occurs around 5 days of age. Spermatogenesis reaches the pachytene stage at 20 days of age, round spermatids are seen at 25 days of age, and mature spermatozoa are seen free in seminiferous tubules at the age of 50 days (Clermont and Perey, 1957). Data on antioxidant enzyme activity in different testicular cells has been reported. Sertoli cells express considerable SOD and GSH-Tr activity, and pachytene spermatocytes and round spermatids express even higher levels of SOD activity, but no measurable catalase or GSH-Px activity (Yoganathan et al, 1989a). Hexose monophosphate shunt activity has been reported to be higher in Sertoli cells than in germ cells (DenBoer et al, 1990). Relatively high levels of GSH-Tr activity have been found in rat peritubular cells and in Leydig tumor cells (Yoganathan et al, 1989b). Considering these data, the decline in GSH-Px and HMS activity could be at least partly due to the age-related increase in the proportion of testicular germ cells. More information is needed to determine the role of different testicular cell populations in the increase of GSH-Tr and catalase activity. Pradeep et al (1990) have reported an alternate luteinizing hormone-inducible pathway of testosterone biosynthesis mediated by SOD. The drop in SOD activity by 20 days of age parallels the replacement of fetal-type Leydig cells by adult-type cells and

the concomitant decline in testosterone production (Tapanainen et al, 1984). However, as the relative number of Leydig cells is low even at 6 to 10 days of age (Tapanainen et al, 1984), the change in the Leydig cell population seems not to be the sole explanation for the very high level of SOD activity at that age and the sharp drop thereafter. Other changes in the relative proportions of different cells hardly explain this sudden drop, either.

Testicular descent in the rat occurs around 21 days of age. It is an androgen-dependent process (Rajfer and Walsh, 1977) in which abdominal pressure plays a role (Frey et al, 1983). Superoxide dismutase activity in the testis has been reported to be reduced by elevated temperature (37°C or 40°C) and by experimental cryptorchidism (Ahotupa and Huhtaniemi, 1992). This finding is in contrast with a drop in SOD activity at the descent of the testes, and the testes of the 20-day-old rats were probably still undescended. Yet, testicular descent may be involved in the increase in catalase activity and the decrease in HMS activity between 20 days and 1 month of age.

The physiologic reason for high SOD activity in the young age groups could be an increased need for hydrogen peroxide in some as yet unidentified developmental processes. Hydrogen peroxide has been reported to stimulate prostaglandin production in the rat renal glomeruli (Baud et al, 1981). Prostaglandins have been suggested to be modulators of cell proliferation (Baud and Ardaillou, 1986) and hydrogen peroxide has been hypothesized to be an effector of cell differentiation (Sohal et al, 1986). Previously, SOD has been found to increase during metamorphosis in insects (Fernandez-Souza and Michelson, 1976). In addition, arachidonic acid metabolites are capable of producing reactive oxygen species by stimulating protein kinase C (McPhail et al, 1984; Badwey et al, 1984). In the rat testis, active protein kinase C has been found to peak at the age of 10 days (Eskola et al, unpublished data). Thus, high SOD activity is suggested to lead to stimulation of protein kinase C and to a positive feedback effect on itself via reactive oxygen species.

The present study demonstrates that there are considerable changes in the antioxidant enzyme system in the maturing rat testis. The meaning of these developmental profiles remains to be seen, but the high level of SOD activity at an early age could contribute to the suggested physiologic role of hydrogen peroxide in differentiation processes. Moreover, these data on antioxidant enzyme activities may help to explain the susceptibility of the testis to oxidative stress.

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