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Effects of Estradiol Benzoate and Progesterone on Superoxide Dismutase Activity in the Thymus of Rats

J. KASAPOVIĆ, S.B. PAJOVIĆ, S. PEJIĆ, J.V. MARTINOVIĆ

Laboratory for Molecular Biology and Endocrinology, Vinča Institute for Nuclear Sciences, Belgrade, Yugoslavia

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Summary

The activity of mitochondrial superoxide dismutase (MnSOD) and cytosol superoxide dismutase (CuZnSOD) was measured in corresponding subcellular fractions prepared from the thymi of intact and chronically gonadectomized (GX) rats of both sexes, as well as of GX male and female rats injected subcutaneously with a single dose of 5 μg estradiol benzoate (EB) and/or 2 mg progesterone (P). Animals were sacrificed 2 h or 24 h following hormone treatment. In the females, the activity of MnSOD in the thymus was stable during the estrous cycle and did not change after ovariectomy. Treatment of GX females with estradiol benzoate resulted 2 h later in a significant elevation of MnSOD activity, whereas 24 h later the activity returned back to control values. On the other hand, treatment of GX females with progesterone had no effect on the MnSOD activity. However, combined hormone treatment, in which EB injection preceded progesterone injection by one hour, enhanced the effect on MnSOD activity similar to that of estradiol benzoate alone. The activity of CuZnSOD in cycling rats was increased in proestrus, whereas removal of the ovaries kept the values at low diestrus and estrus levels. Contrary to MnSOD, CuZnSOD activity did not change after EB treatment of GX females, while progesterone increased the enzyme activity at 2 h and 24 h after hormone treatment. However, combined EB+P treatment proved to be ineffective. In the males, neither MnSOD nor CuZnSOD activity was affected by the removal of testes or by progesterone treatment of GX animals. Only EB injection to GX rats significantly increased CuZnSOD activity 24 h later.

Key words

Superoxide dismutase • Thymus • Progesterone • Estradiol • Rats

Introduction

Growing evidence suggests that the activity of superoxide dismutases (SODs), the enzymes protecting cells from oxygen toxicity by catalysing the dismutation of superoxide radicals ($O_2^{\bullet-}$) in H_2O_2 and O_2, can be modulated by sex steroids in various target tissues. Thus, it was shown that the chronic treatment of male Syrian hamsters by diethylstilbestrol causes temporary increase of MnSOD activity in the kidney (McCormick *et al.* 1991). In cycling rats, the activity of MnSOD in the brain (Pajović *et al.* 1993) and the total SOD in the ovary (Laloraya *et al.* 1988) is increased in the proestrus, while the activity of total SOD in erythrocytes is stable during the cycle (D'Almeida *et al.* 1995). Estrogen treatment does not cause significant changes of the total SOD activity in the kidney and liver of hamsters (Roy and Liehr 1989). It was also shown that the level of $O_2^{\bullet-}$ in the ovary varies cyclically during the estrous

Fax+4202 24920590
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

cycle (Laloraya *et al.* 1988), and that changes of physiological concentrations of estradiol, progesterone and testosterone significantly alter the production of $O_2^{\cdot-}$ and H_2O_2 in macrophages of rats (Chao *et al.* 1994).

The modulation of other antioxidant enzymes by sex steroids has also been reported. The activity of glutathione peroxidase in the liver of rats is the highest in estrus and is significantly increased during pregnancy (Pinto and Bartley 1969). The activity of this enzyme in the blood of rats is significantly increased during the early proestrus and late diestrus and correlates with the level of progesterone (Smith *et al.* 1995). Therapeutic substitution with 17β-estradiol restores lowered activity of glutathione peroxidase in erythocytes of amenorrhoic female patients, whereas progesterone is without effect (Massafra *et al.* 1997).

Gonadotropins are also possible modulators of SOD activity. Thus, luteinizing hormone (LH) increases SOD activity in the ovary of rats (Laloraya *et al.* 1988), pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) lower MnSOD, but do not influence CuZnSOD in the ovary of rats (Sato *et al.* 1992), and hCG increases SOD activity in human corpora lutea (Vega *et al.* 1994).

We have shown that estradiol and progesterone suppress MnSOD activity in the brain of rats and do not influence the activity of CuZnSOD (Pajović *et al.* 1993, 1996). In the liver, these hormones demonstrate an opposite effect on CuZnSOD activity and do not influence the activity of MnSOD (Kasapović *et al.* 1997). Also, it was reported that olive oil has a modulatory role on SOD activity in rat tissues (Pajović *et al.* 1997, Pejić *et al.* 1999) and that thymus hypocholesterolemic factor, which participates in cholesterol metabolism, has identical amino acid sequences and antioxidant function as bovine erythrocyte SOD (Mondola *et al.* 1993). These data could show a complex interaction between sex steroid metabolism and SOD activity within the lipid metabolic pathways.

The aim of this study was to examine whether sex steroids play a role in the modulation of antioxidative protection in the thymus, besides their well established role in the control of development and maintenance of the immunological and endocrine function of this organ (Pierpaoli and Sorkin 1972, Clarke and Kendall 1994). Therefore, we examined the effects of estradiol benzoate and progesterone on the activity of MnSOD and CuZnSOD in the thymus of rats of both sexes. We postulated that these data may provide additional evidence about the importance of sex steroids in the regulation of $O_2^{\cdot-}$ and H_2O_2 levels, which were proven to play the role of secondary messengers (Riley and Behrman 1991, Behrman *et al.* 1993) and participate in the antimicrobial protection of phagocyte cells (Chao *et al.* 1994) and apoptotic death of thymocytes (Briehl *et al.* 1995, Briehl and Baker 1996) .

Methods

Wistar rats aged 3.5 months, with average weight of 265 g for female and 328 for male rats, were used. They were housed in open colony cages (6 animals per cage) under controlled conditions of temperature (23±2 °C) and illumination (lights on from 5:00 to 17:00 h), and had a free access to tap water and laboratory chow. At sacrifice, intact females were classified as proestrous, estrous and diestrous according to their vaginal smears.

In hormone replacement experiments, long-term gonadectomized male and female animals were used. Bilateral gonadectomy was performed under ether anesthesia 3 weeks (females) or 2 weeks (males) prior to the experiments.

Each gonadectomized (GX) female and male animal received a single s.c. dose of 5 μg estradiol benzoate (β-estradiol-3-benzoate, Sigma) or 2 mg progesterone (Sigma), suspended in 0.1 ml olive oil. Control GX animals received 0.1 ml olive oil by the same route. The same hormone and vehicle amounts were used in the combined EB+P treatment, in which estradiol benzoate was given 1 h prior to P. Treated GX females were sacrificed 2 h or 24 h, and GX males 24 h, after hormonal treatment.

All animals were killed between 10:00 and 10:30 h in the morning by decapitation with a guillotine (Harvard Apparatus) and fresh thymi were dissected for sample preparations. Tissue homogenates were prepared according to a slightly modified methods of Rossi *et al.* (1983) and De Waziers and Albrecht (1987). The thymus tissue was homogenized in 0.05 M KH_2PO_4 buffer containing 0.1 mM EDTA, pH 7.8. Homogenates were vortexed for 30 s several times with intermittent cooling on ice and left frozen at –70 °C for 20 h. The homogenates were then defrosted and centrifuged at 37 000 rpm for 65 min. Cytosols were kept at –20 °C until use. Protein concentration in the cytosol was determined by the method of Lowry *et al.* (1951).

Enzymatic activity of SOD was determined by the method of Misra and Fridovich (1972) before and after the inhibition of CuZnSOD with KCN (Geller and Winge 1983). This method is based on the ability of SOD to inhibit

the autoxidation of adrenaline into adrenochrome at pH 10.2. Inhibition of autoxidation was monitored at 480 nm. The enzyme activity was expressed in units per mg protein. One unit of SOD was defined as the amount of protein that caused 50 % inhibition of conversion rate of adrenaline to adrenochrome between the third and the fourth minute of incubation. The results were analyzed by Student's t-test and ANOVA in combination with Scheffe's procedure.

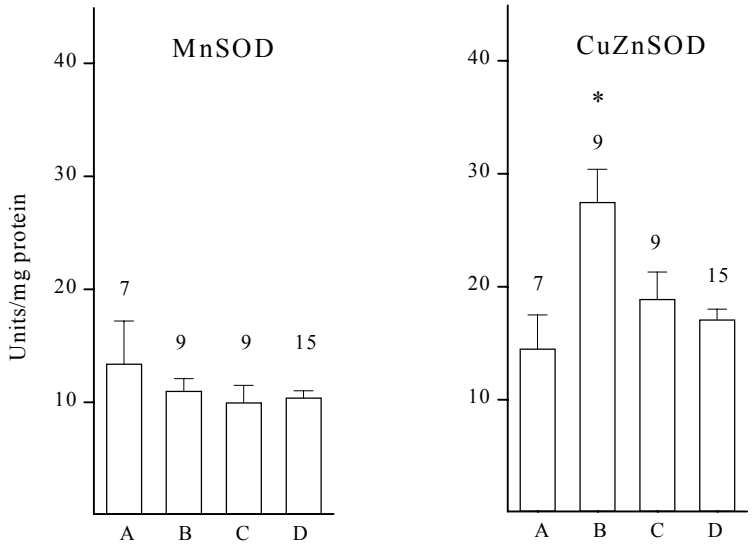

Fig. 1. *SOD activities in the thymus of female rats at diestrus (A; n=7), proestrus (B; n=9) and estrus (C; n=9) and following ovariectomy (D; n=15). Data are means ± S.E.M. The number of samples is indicated above the respective columns.* * *p<0.05 (ANOVA).*

Results

MnSOD and CuZnSOD activities in cycling and ovariectomized (OVX) rats

During the estrous cycle (Fig. 1), the activity of MnSOD was steady (diestrus: 13.4±3.8 units/mg protein; proestrus: 11.0±1.1; estrus: 10.0±1.5; $F_{2,22}$ = 0.610, p>0.05), whereas CuZnSOD activity was significantly elevated ($F_{2,22}$ = 7.369, p<0.05) in the morning of proestrus (27.5±2.9) in comparison to diestrus (14.5±1.3) and estrus (18.9±2.4).

Removal of the ovaries (Fig. 1) did not affect the activity of MnSOD (10.4±0.6; $F_{3,36}$ = 0.702, p>0.05). At the same time, CuZnSOD activity (17.1±0.9; $F_{3,36}$ = 8.088, p<0.05) was significantly reduced in comparison to the proestrus values.

Effect of estradiol benzoate on MnSOD and CuZnSOD activities in OVX rats

Treatment of OVX animals with estradiol benzoate (Fig. 2) induced a significant elevation of MnSOD activity 2 h later (OVX controls: 7.0±1.0; OVX+EB: 14.1±1.1; t_{13} = 4.779, p<0.05), which returned back to control levels 24 h after the hormone treatment (OVX controls: 11.6±1.5; OVX+EB: 13.2±3.5; t_{22} = 0.473, p>0.05).

The activity of CuZnSOD in OVX animals treated with estradiol benzoate remained unaffected 2 h later (OVX controls: 17.4±1.9; OVX+EB: 19.7±4.8; t_{13} = 0.503, p>0.05) and 24 h later (OVX controls: 26.1±3.2; OVX+EB: 20.7±3.1; t_{22} = 1.129, p>0.05).

Effects of progesterone on MnSOD and CuZnSOD activities in OVX rats

Systemic application of progesterone to OVX rats (Fig. 2) did not affect MnSOD at either 2 h (OVX controls: 7.0±1.0; OVX+P: 8.2±2.3; t_{14} = 0.557, p>0.05) or 24 h after hormone treatment (OVX controls: 11.6±1.5; OVX+P: 11.1±0.6; t_{23} = 0.280, p>0.05). In contrast, profoundly elevated activity of CuZnSOD was found both at 2 h (OVX controls: 17.4±1.9; OVX+P: 31.35±3.3; t_{14} = 3.886, p<0.05) and 24 h (OVX controls: 26.1±3.2; OVX+P: 36.5±1.6; t_{23} = 2.479, p<0.05) after hormone treatment.

Effects of estradiol benzoate and progesterone on MnSOD and CuZnSOD activities in OVX rats

Two hours after successive injections of estradiol benzoate and progesterone given to OVX animals, the activity of MnSOD was increased (OVX controls: 5.7±0.9; OVX+EB+P: 9.8±0.8; t_{15} = 3.349, p<0.05) and that of CuZnSOD remained unchanged (OVX controls: 21.9±2.5; OVX+EB+P: 20.2±2.9; t_{15} = 0.432, p>0.05) in respect to control values (Fig. 3).

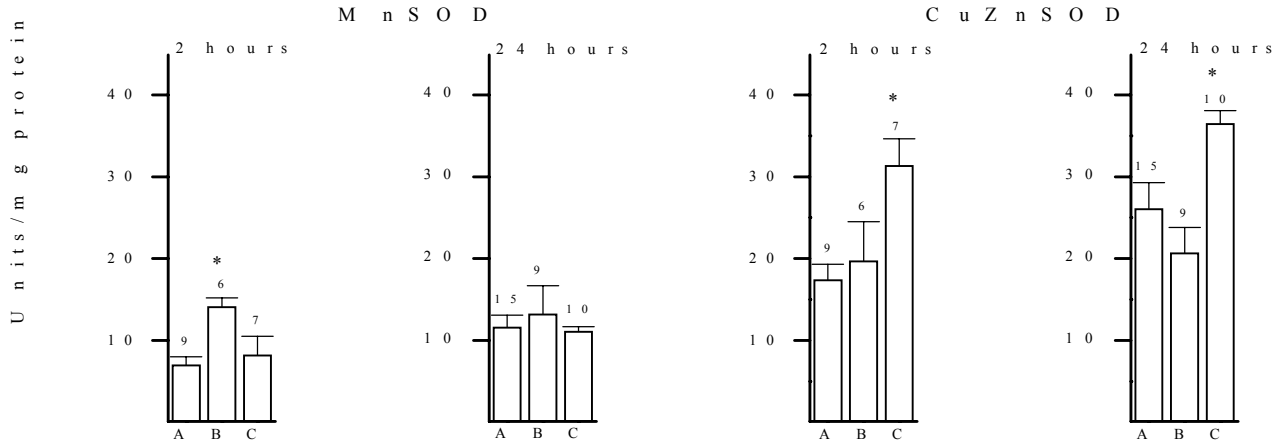

Fig. 2. *SOD activities in the thymus of ovariectomized rats, sacrificed 2 h or 24 h following treatment with olive oil (A; n=9 at 2 h; n=15 at 24 h), estradiol benzoate (B; n=6 at 2 h; n=9 at 24 h) and progesterone (C; n=7 at 2 h; n=10 at 24 h). Data are means ± S.E.M. The number of samples is indicated above the respective columns.* * *p<0.05 (ANOVA for A, B and C).*

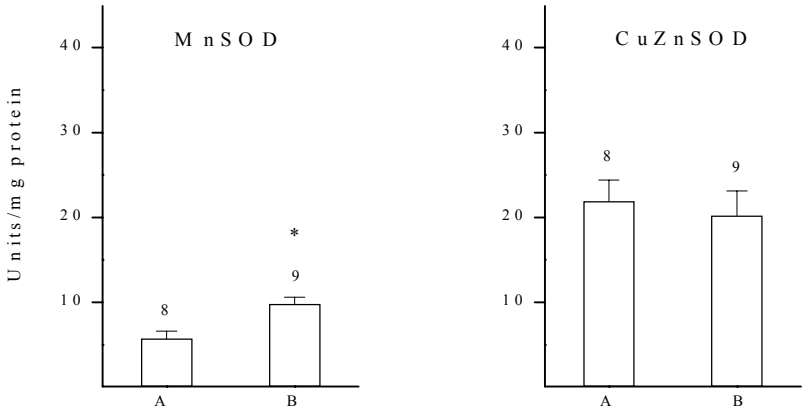

Fig. 3. *SOD activities in the thymus of ovariectomized rats, sacrified 2 h following treatment with olive oil (A; n=8), estradiol benzoate plus progesterone (B; n=9). Data are means ± S.E.M. The number of samples is indicated above the respective columns.* * *p<0.05 (t-test).*

MnSOD and CuZnSOD activities in intact and GX male rats

Intact male rats did not differ from chronically castrated animals with respect to both MnSOD (intact: 10.1±0.6; GX: 11.2±0.7; $t_9 = 1.233$, $p>0.05$) and CuZnSOD activity (intact: 18.1±2.0; GX: 19.9±1.6; $t_9 = 0.658$, $p>0.05$) in the thymus (Fig. 4).

Effect of estradiol benzoate and progesterone on MnSOD and CuZnSOD activities in TX rats

In comparison to GX controls (10.3±0.8), the activity of thymus MnSOD was not changed in GX animals treated 24 h earlier with estradiol benzoate (11.3±0.9; $t_{10} = 0.805$, $p>0.05$) or with progesterone (8.7±0.6; $t_{10} = 1.703$, $p>0.05$). Similarly, treatment of GX animals with progesterone did not affect the activity of CuZnSOD (16.5±1.6; $t_{10} = 0.078$, $p>0.05$), but estradiol benzoate significantly enhanced the activity of this enzyme (21.7±1.2; $t_{10} = 3.237$, $p<0.05$), as compared to the GX controls (16.3±1.1) (Fig. 4).

Discussion

The activity of thymus CuZnSOD is increased in proestrus, while after the removal of gonads it is significantly decreased compared to the proestrus value.

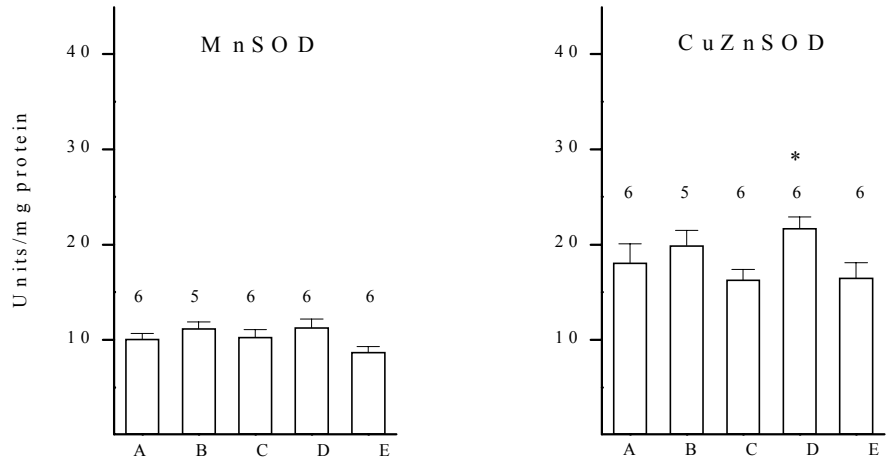

Fig. 4. *SOD activities in the thymus of male rats, intact (A; n=6), gonadectomized (GX) non-treated (B; n=5), GX treated with olive oil (C; n=6), GX treated with estradiol benzoate (D; n=6) and GX treated with progesterone (E; n=6). Data are means ± S.E.M. The number of samples is indicated above the respective columns.* * *p<0.05 (ANOVA for C, D and E).*

This suggests the direct or indirect effect of ovarian hormones on the cytosol SOD activity. Single treatment of OVX females with estradiol benzoate is without effect, while progesterone stimulates the activity of CuZnSOD. It is interesting that the combined hormone treatment does not change the activity of this enzyme, which shows that EB, although without its own effect, eliminates the stimulative effect of progesterone on CuZnSOD. It is possible that this indirect influence of estradiol benzoate seen in OVX females increases CuZnSOD activity in proestrus, when plasma levels of estradiol are high and progesterone is low (Clemens and Weaver 1985). Estrogen could exert its influence on progesterone-induced change of CuZnSOD activity on the level of receptors for progesterone (Kraus *et al.* 1994) or on some other level. The lowered activity of CuZnSOD in GX females compared to the proestrus value could reflect a post-castration increase of circulating gonadotropines (Yamamoto *et al.* 1970, Eldridge *et al.* 1974). Thus, the change of thymus CuZnSOD in OVX animals could be the consequence of the direct effect of progesterone or its indirect effect *via* the gonadotropines. This possibility arises from the findings that LH (Laloraya *et al.* 1988) and hCG (Sato *et al.* 1992, Vega *et al.* 1994) specifically modify the SOD activity in the ovary. Since we have observed the effects of ovarian hormones on both types of SOD 2 h after a single dose, which was different than after the 24-hour treatment, we have chosen the combined 2-hour treatment. Prolonged effect of progesterone as well as a combined effect of estradiol benzoate and progesterone on cytosol SOD could be physiologically significant, judged by the altered activity of this enzyme during the estrous cycle and after gonadectomy. On the other hand, in GX male thymi, the effect of estradiol benzoate on CuZnSOD activity does not seem to be physiologically relevant since elimination of endogenous steroids by castration of males has no influence on this enzyme.

The activity of thymus MnSOD in OVX females is elevated 2 h after EB treatment. Since progesterone is without effect, the increased activity of this enzyme after the combined hormone treatment is the consequence of stimulation by EB. The acute effect of estradiol benzoate disappears during the period of 2-24 h after treatment, which is in agreement with the stability of MnSOD activity during estrous cycle and after ovariectomy and it seems not to be physiologically relevant. A similar stability of MnSOD activity in relation to the hormone treatment was observed in males and females 24 h after treatment, whereas modulation of cytosol SOD was specific in relation to the type of ovarian hormone. In the experiments on males we have chosen the 24 h period because it represents a period long enough for attaining the effects of estradiol benzoate and progesterone as well as of their metabolites.

The results presented in this work suggest that ovarian steroids could modulate the activity of cytosol SOD in the thymus of female rats and together with the previously described effects in the rat brain (Pajović *et al.* 1993, 1996) and liver (Kasapović *et al.* 1997) show that this modulation is tissue-specific. Taking into consideration that free radicals have an important role in processes of phagocytosis and apoptosis, it is obvious that ovarian hormones may have an influence on these processes in the thymus by modulating SOD activity. Furthermore, since sex steroids greatly influence the thymus physiology during

pregnancy (Clarke and Kendall 1994), we expect to get more information about their SOD-modulatory role in future experiments with chronic estradiol benzoate and progesterone treatments as well as on pregnant females.

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Reprint requests

Dr. Snežana B. Pajović, Laboratory for Molecular Biology and Endocrinology, Vinča Institute for Nuclear Sciences, P.O.Box 522, 11001, Belgrade, Yugoslavia. Fax.: +38-1-11-455-561, e-mail pajovic@rt270.vin.bg.ac.yu