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Antioxidant system in rat testicular cells

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Abstract

The activity of the enzymes involved in the antioxidant defence – superoxide dismutase (SOD), glutathione peroxidase (GPx), reductase (GR), *S*-transferase (GST) – as well as the glutathione (GSH) levels were measured in different rat testicular cell populations. A differential distribution of these components among testicular cell types was clearly observed. Sertoli and peritubular cells had elevated SOD and GSH-dependent enzyme activities associated with a high GSH content. Compared with the somatic cells, pachytene spermatocytes (PS) and round spermatids (RS) presented a different antioxidant system characterized by higher SOD activity and GSH content associated with very low GSH-dependent enzyme activity. Spermatozoa exhibited the same enzymatic system as PS and RS but were devoid of GSH. Interstitial tissue displayed high GSH content, moderate SOD and GSH-related enzyme activity except for GPx which was very elevated. It is concluded that the different categories of testicular cells probably display a highly variable susceptibility to oxidative stress.

Key words: Testicular cell; Glutathione; Antioxidant enzyme; Reactive oxygen; Rat

1. Introduction

Reactive oxygen species, including superoxide (O_2^-), singlet oxygen (O_2^1), hydroxyl radical (OH), hydrogen and organic peroxides (H_2O_2, ROOH), are overproduced by cells exposed to radiation [1] or redox-cycling drugs [2]. These intermediates are known to be cytotoxic and often cause tissue injury in diseases, such as atherosclerosis, cancer, rheumatoid arthritis, and in a variety of situations, such as ischemia/reperfusion, hyperoxia, chemo- and radiotherapeutic treatment [3]. O_2^-, peroxides are postulated to be relatively inactive, and much of their toxicity is considered to result from their subsequent metal ion catalyzed formation of the highly reactive OH [4]. The hydroxyl radical is a very powerful oxidant that can attack all types of biological molecules, including lipids [5], DNA [6,7] and proteins [8].

Cells are normally protected against oxidative damage by multiple enzymatic mechanisms and by antioxidant molecules. The first enzymes involved, cytosolic Cu/Zn and mitochondrial Mn superoxide dismutases (SOD), scavenge O_2^- by converting it to H_2O_2 [9], thus preventing cell injury mediated by oxygen radicals, in various circumstances [10–15]. Hydrogen peroxide is then metabolized, either by the peroxisomal catalase or, to a greater extent, by cytosolic and mitochondrial seleno-glutathione peroxidase (Se-GPx) [16]. Organic peroxides are reduced by the latter enzyme [16] and also, specifically, by selenium-independent enzymes named glutathione *S*-transferases (GST) [17,18]. It has been suggested that Se-GPx [19–21] and GST [22,23] play a key role in cellular defence against oxidative damage.

When peroxides are detoxicated by the different GSH peroxidases (GPx), glutathione (GSH) is converted to oxidized glutathione (GSSG). This disulfide is subsequently reduced by a NADPH-dependent enzyme, the glutathione reductase (GR), thus maintaining the GSH/GSSG ratio. Several studies have also demonstrated an important role of this enzyme in cellular resistance to reactive oxygen species [24,25].

Taken together, these different observations suggest that, to efficiently protect the cell against oxidative stress, a concerted detoxification of O_2^- and of hydrogen or organic peroxides by SOD, Se-GPx, GST and GR, is necessary. These enzymes, working simultaneously, probably prevent the formation of the highly cytotoxic hydroxyl radical.

Among the various existing antioxidant molecules, glutathione appears to be a key component. This tripeptide has different functions which contribute to cell defence and protection: free radical scavenger, coenzyme for several antioxidant enzymes, maintenance of the thiol–disulfide status, detoxication of electrophilic xenobiotics via conjugation [26]. The importance of glutathione in protecting various cells against free radical injury or chemically induced damage is now well established [26–28].

Although the testis is very sensitive to the deleterious effects of various chemical agents [29] and radiation [30], the different antioxidant systems in testicular cell populations have not been intensively studied. Previous reports have indicated the presence of SOD [31], GSH [32–35] and of some GSH-dependent enzymes in the testis, including GST [31,35–37], Se-GPx [31,35,38], GR [35–39]. The exact cellular location of these different components is, however, far from being established. Therefore, the present study aimed at gaining more information about the defence capacity of the somatic and germinal testicular cells by measuring the GSH content,

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as well as the SOD and GSH-dependent enzyme activity in different rat testis cell types.

2. Materials and methods

2.1. Chemicals

Culture media (Ham's F12, DMEM, PBS, Dulbecco) were obtained from Gibco (Cergy Pontoise, France). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Sprague–Dawley male rats, aged 20 or 90 days, were provided by the Janvier Breeding Centre (Le Genest, France). They were housed under normal laboratory conditions in a 12 h light/12 h dark cycle, fed standard commercial food and given water ad libitum.

2.3. Enriched cell preparations

Peritubular and Sertoli cells from 20-day-old Sprague–Dawley rats were prepared as described by Mather and Phillips [40] and cultured for 3 days (10^6 cells/ml) in Ham's F12/DMEM medium (1 : 1 v/v) supplemented with gentamicin (4 μg/ml), insulin (5 μg/ml) and transferrin (5 μg/ml) at 32°C in a humidified atmosphere of 95% air/5% CO_2. The peritubular cells were allowed to adhere to the plastic substrate, after which they were rinsed several times to remove contaminating germ cells. On day 2 of culture, the Sertoli cell monolayers were subjected to a hypotonic shock (20 mM Tris-HCl buffer solution, pH 7.4) for 2 min to remove the remaining contaminating germ cells [41], and then incubated for a further 24 h in the supplemented Ham's F12/DMEM medium. At the end of the 3-day culture, the medium was removed, the cells were rinsed with PBS and frozen at −80°C in a potassium phosphate buffer (0.1 M, EDTA 1 mM, pH 7.0) until enzymatic assays were performed. Using this procedure, the Sertoli cells were contaminated less than 2% by germ cells.

Pachytene spermatocytes (PS) and round spermatids (RS) were isolated from 90-day-old Sprague–Dawley rat testes by centrifugal elutriation, as previously described [42]. Enrichment of the PS and RS fractions evaluated by DNA flow cytometry ranged from 80 to 85%, respectively. Germ cell viability assessed by the Erythrosine red exclusion test was equal or superior to 95%.

The cauda epididymis from 90-day-old rats was excised, rinsed and dispersed in PBS. The fragments were allowed to sediment, the supernatant was then centrifuged (100 × *g*, 10 min) and the pelleted spermatozoa were collected.

Interstitial cells were obtained by collagenase digestion of decapsulated testes from 90-day-old rats, as previously described [43]. After filtration through a nylon filter (pore size 20 μm), cell suspensions were pelleted by centrifugation (100 × *g*, 10 min).

All the cell fractions were frozen at −80°C in a potassium phosphate buffer containing 0.1 M EDTA at pH 7.0.

2.4. Enzyme assays

After thawing, the different testicular cell preparations were sonicated for 3 × 10 s at 0°C using a micro-ultrasonic cell disrupter. Sonicates were centrifuged for 10 min at 1,000 × *g* and supernatants were used for enzyme assays. Supernatant protein content was determined by the Lowry method with bovine serum albumin as a reference [44].

Total SOD activity (mitochondrial Mn SOD + cytosolic Cu/Zn SOD) was evaluated spectrophotometrically by monitoring at 550 nm the inhibition of the reduction of ferricytochrome *c*, using the xanthine–xanthine oxidase system as the source of O_2^- [45]. One unit of SOD is defined as the amount of the enzyme required to inhibit 50% of the rate of cytochrome *c* reduction.

GPx activity was measured at 37°C by recording the oxidation of NADPH at 340 nm in the presence of GSH and glutathione reductase [46]. Se-GPx activity was evaluated with H_2O_2 as the substrate, in the presence of NaN_3 to inhibit the catalase, while total GPx activity (Se-GPx plus Se-independent GPx) was determined using *t*-butyl hydroperoxide. Enzymatic activity is expressed as nmol of NADPH oxidized/min/mg protein (ε_{340} = 6.22 $mM^{-1} \cdot cm^{-1}$).

GR activity was determined at 37°C by monitoring the oxidation of NADPH at 340 nm [47]. Enzymatic activity is expressed as nmol of NADPH oxidized/min/mg protein (ε_{340} = 6.22 $mM^{-1} \cdot cm^{-1}$).

GST activity was measured at 30°C by determining spectrophotometrically at 340 nm the rate of conjugate formation between GSH and 1-chloro-2,4-dinitrobenzene [48]. Enzyme activity is expressed as nmol of S-2,4-dinitrophenylglutathione formed/min/mg protein (ε_{340} = 9.6 $mM^{-1} \cdot cm^{-1}$).

2.5. Glutathione determination

The supernatants obtained after sonication of the different cell fractions were deproteinized by adding 5-sulfosalicyclic acid (5%, w/v). After standing on ice, the samples were centrifuged (1,000 × *g*, 10 min) and total glutathione (GSH and GSSG) was determined by the glutathione disulfide reductase-5,5′ dithiobis (2-nitrobenzoic acid) recycling assay [49]. The rate of 5-thio-2 nitrobenzoic acid formation was followed at 412 nm at 30°C. The amount of total glutathione was determined from the standard curve simultaneously obtained under the same conditions with standard solutions of GSH.

2.6. Statistical analysis

Data were expressed as the mean ± S.E.M. for the number of separate experiments indicated. Differences between the testicular cell types were analyzed using Student's *t*-test for unpaired data. $P < 0.05$ was selected as the threshold of statistical significance.

3. Results

3.1. Activity of SOD (Mn SOD plus Cu/Zn SOD)

SOD activity displayed marked differences between somatic testicular cells and the germinal cell types. The lowest SOD activity was measured in interstitial, peritubular and Sertoli cells (Fig. 1). In contrast, PS, RS and spermatozoa contained 38–56% more activity when compared to the latter cells.

3.2. Activity of GSH-dependent enzymes

The highest Se-dependent GPx activity occurred in interstitial cells, whereas the lowest activity was measured in PS. Sertoli cells, peritubular cells, RS and spermatozoa presented comparable, intermediate Se-GPx levels (Fig. 2a). An elevated total GPx activity (Se-GPx

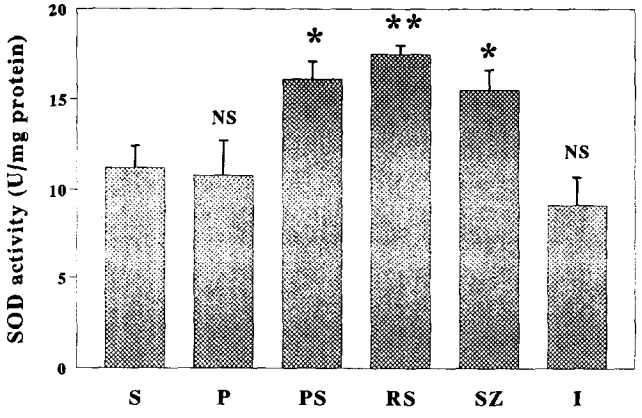

Fig. 1. Total superoxide dismutase (SOD) activity in rat testicular cells and epididymal spermatozoa. SOD activity was measured in Sertoli cells (S), peritubular cells (P), pachytene spermatocytes (PS), round spermatids (RS), spermatozoa (SZ) and interstitial cells (I), as described in section 2. Results are expressed as U/mg protein and are means ± S.E.M. for 4 separate preparations. * $P < 0.05$ by Student's *t*-test as compared to Sertoli cell activity. NS, not significant.

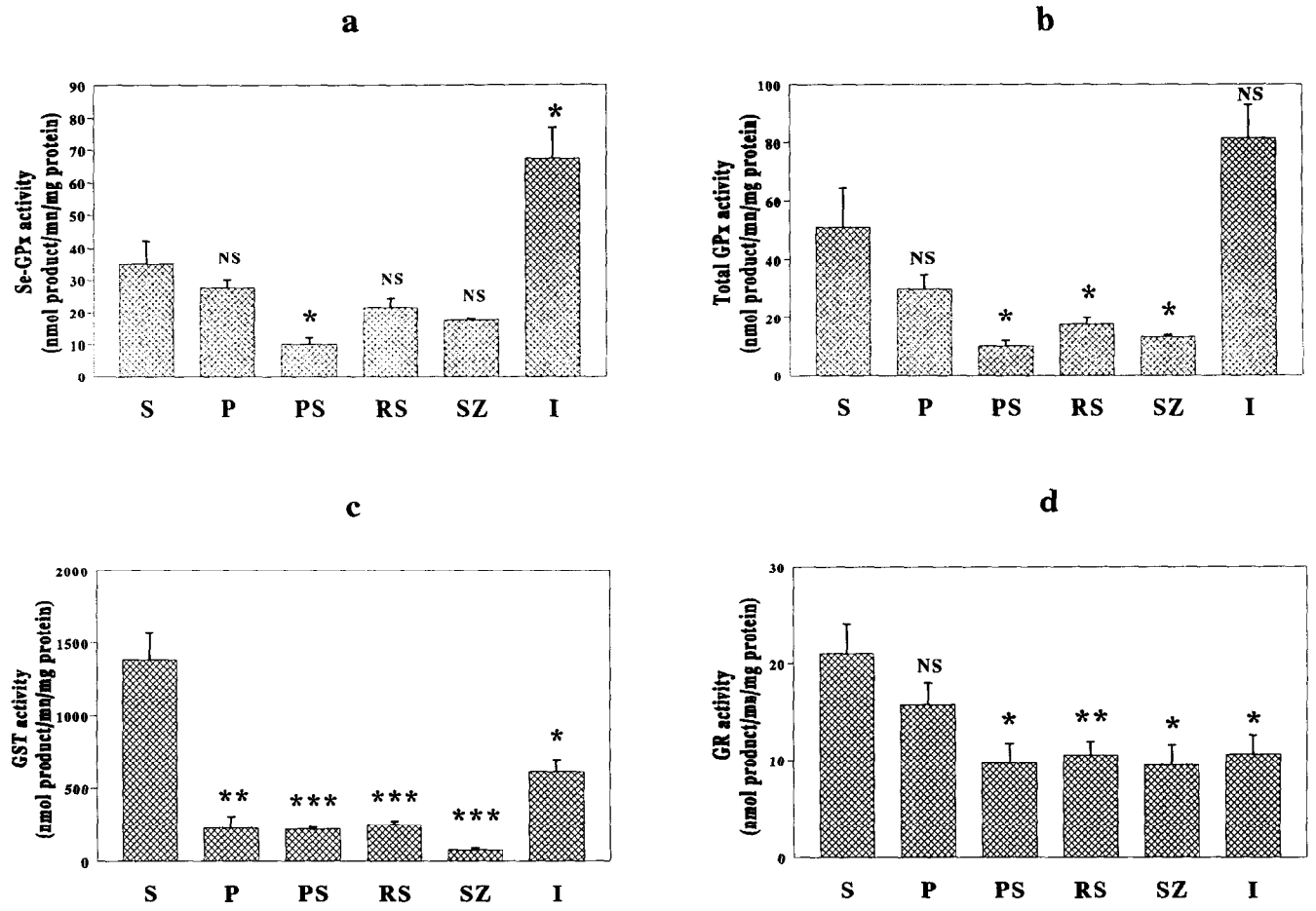

Fig. 2. GSH-dependent enzyme activity in rat testicular cells and epididymal spermatozoa. Se-GPx (a), total GPx (b), GST (c) and GR (d) activities were determined in Sertoli cells (S), peritubular cells (P), pachytene spermatocytes (PS), round spermatids (RS), spermatozoa (SZ) and interstitial cells (I) as described in section 2. Data shown are expressed as nmol of product formed/min/mg protein and represent means ± S.E.M. for 4 separate preparations. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Student's t-test, as compared to activities measured in Sertoli cells. NS, not significant.

plus Se-independent GPx) was found in interstitial, Sertoli and peritubular cells, whereas the lowest values were observed in the different germ cell fractions and in spermatozoa (Fig. 2b). GST activity was highest in Sertoli cells, intermediate in interstitial cells and very low in peritubular cells, PS, RS and spermatozoa (Fig. 2c). Sertoli cells were found to contain significantly more GR when compared to the different germ cell fractions and to interstitial cells (Fig. 2d). The GR activity in peritubular cells was not significantly different from that measured in Sertoli cells.

3.3. GSH content

GSH levels varied markedly among the different cell types (Fig. 3). The GSH content was identical for isolated PS and RS and about double that of Sertoli and peritubular cells; interstitial cells presented intermediate levels. GSH was undetectable in spermatozoa which agrees with previous studies [33,34].

4. Discussion

Data on antioxidant enzyme activity in whole rat and guinea pig testes [31,50] have been previously reported. As compared to the liver, rat testes express equivalent SOD activity but only 5% of the GPx liver levels and 2% of the liver catalase activity [31]. Testicular guinea pig SOD levels were found to be about twice as high as liver SOD in the same species, whereas GPx, GR and catalase were about 60, 5, 300 times less, respectively [50]. The previous studies on testicular tissues did not investigate the precise cellular distribution of these enzymatic activities. The aim of the present study was, therefore, to evaluate the antioxidant defence capacity of the different categories of testicular cells. Cellular resistance to oxidative agents is controlled by several key enzymes, such as SOD, GSH-dependent enzymes including Se-GPx, Se-independent GPx (GST), GR and by some antioxidant molecules such as GSH. Measuring the activity of these

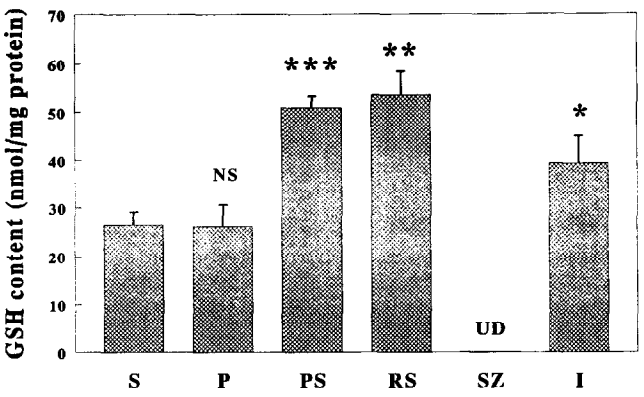

Fig. 3. GSH content of rat testicular cells and epididymal spermatozoa. Total GSH content (GSH + GSSG) was determined in Sertoli cells (S), peritubular cells (P), pachytene spermatocytes (PS), round spermatids (RS), spermatozoa (SZ) and interstitial cells (I) as described in section 2. Results are expressed as nmol of GSH/mg protein and are means ± S.E.M. for 4 separate preparations. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Student's t-test, as compared to activities measured in Sertoli cells. NS, not significant; UD, undetectable.

enzymes and the GSH level in the different testicular cell populations is therefore crucial to understanding testicular antioxidant defence mechanisms.

Each testicular cell type tested is equipped with antioxidant enzymes and molecules. However, distribution of the antioxidative factors clearly depended on the cell type under consideration. The Sertoli cell antioxidant system is characterized by relatively high levels of SOD, Se-GPx, Se-independent GPx, GST, GR activity and intracellular GSH. Sertoli cells should, therefore, be able to convert O_2^- to H_2O_2, to further metabolize this peroxide or other organic peroxides to unreactive molecules, and to maintain the GSH/GSSG ratio. These somatic testicular cells appear to have a complete antioxidative defence system and can probably protect themselves efficiently against oxidative stress. Phagocytosis is always associated with a respiratory burst in which high levels of O_2^- are produced [51]. This feature is particularly important in Sertoli cells because they phagocytose germ cell debris and considerable amounts of late spermatid residual cytoplasm during normal spermatogenesis, and a large number of degenerated germ cells after injury to the testis [52]. Protection against reactive oxygen species is, therefore, essential for Sertoli cells under normal and pathological conditions.

In terms of antioxidative equipment, no significant difference was observed between Sertoli and peritubular cells, except for GST activity, which was much lower in the latter.

In contrast, the proportion of antioxidant enzymes in the germ cells studied (PS and RS) was different from that in the Sertoli and peritubular cells. A striking feature of the germ cell antioxidant system is a very high SOD activity associated with low GPx, GR and GST activity. This finding indicates that PS and RS are able to convert O_2^- to H_2O_2, whereas they may encounter major difficulties to further metabolize hydrogen peroxide, as well as organic peroxides, to unreactive molecules. This is further supported by the fact that we did not detect any catalase activity in these germ cell categories (data not shown). The difference between SOD and GSH-dependent enzyme activity in germ cells may lead to saturation of the protective systems against peroxides. As a result, the H_2O_2 formed may be available for conversion into highly toxic hydroxyl radicals via Fenton-type reactions, thus inducing damage to nucleic acids, proteins and cellular lipids. The consequences can be especially dramatic in the testis, with germ cell mutation and the risk of heritable mutation. Furthermore, the high level of polyunsaturated fatty acids in mammalian testes previously reported [53,54] suggests that the plasma membranes of testicular cells may be greatly susceptible to free radical attack. The fact that germ cells, despite a high GSH level, are not well equipped to combat oxidative stress or xenobiotic-mediated injury probably explains the high sensitivity of these cells to ionizing radiation [30] and to drugs [29] and, also, the extremely limited viability of isolated spermatogenetic cells in culture [55].

Various observations indicate that spermatozoa may be more exposed and vulnerable to oxidative stress than germ cells. First, epididymal spermatozoa are not protected, like PS and RS, by the microenvironment provided by the Sertoli cell barrier. Second, the membranes of spermatozoa may be particularly susceptible to free radical attack because of their high level of polyunsaturated fatty acids [53]. Third, several groups have demonstrated that, in contrast to spermatogonia, PS and RS, elongated spermatids and spermatozoa have a reduced capability or are even unable to repair DNA damage [56,57]. Lastly, contrasting with PS and RS, our results show that spermatozoa are devoid of GSH. This means that the GSH-dependent enzymes involved in the antioxidative defence and detoxication are probably inactive in spermatozoa. We cannot exclude, however, the possibility that spermatozoa may be protected by other antioxidant factors which were not examined here.

The testicular antioxidative defence system is not restricted to the seminiferous tubules. In fact, our results clearly show that the second testicular compartment, the interstitial tissue, also displayed SOD activity, as well as GSH-related enzyme activity, associated with a high GSH content. In addition, it is interesting to note that the most important GSH peroxidase activity was observed in this extratubular compartment. This suggests that the testicular interstitium may be particularly well protected against hydrogen and organic peroxide toxicity. However, since we used crude interstitial tissue preparations in this experiment, further studies are needed to determine which specific cell type (Leydig cells, fibro-

blasts, macrophages or blood cells) accounts for the major GSH peroxidase activity in this compartment.

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