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Aging alters the functional expression of enzymatic and non-enzymatic anti-oxidant defense systems in testicular rat Leydig cells

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Abstract

In aged rats, trophic hormone-stimulated testosterone secretion by isolated Leydig cells is greatly reduced. The current studies were initiated to establish a functional link between excess oxidative stress and the age-related decline in steroidogenesis. Highly purified Leydig cell preparations obtained from 5-month (young mature) and 24-month (old) Sprague–Dawley rats were employed to measure and compare levels of lipid peroxidation, non-enzymatic (α-tocopherol, ascorbic acid, and reduced/oxidized glutathione) and enzymatic (Cu, Zn-superoxide dismutase, Cu, Zn-SOD; Mn-superoxide dismutase, Mn-SOD; glutathione peroxidase-1, GPX-1, and catalase, CAT) anti-oxidants. The extent of lipid perxoidation (oxidative damage) in isolated membrane fractions was quantified by measuring the content of thiobarbituric acid-reactive substances (TBARS) under basal conditions, or in the presence of non-enzymatic or enzymatic pro-oxidants. Membrane preparations isolated from Leydig cells from old rats exhibited two- to three-fold enhancement of basal TBARS formation. However, aging had no significant effect on TBARS formation in response to either non-enzymatic or enzymatic pro-oxidants. Among the non-enzymatic anti-oxidants, the levels of reduced gultathione were drastically reduced during aging, while levels of α-tocopherol and ascorbic acid remained unchanged. Both steady-state mRNA levels and catalytic activities of Cu, Zn-SOD, Mn-SOD, and GPX-1 were also significantly lower in Leydig cells from 24-month-old rats as compared with 5-month-old control rats. In contrast, neither mRNA levels nor enzyme activity of catalase was sensitive to aging. From these data we conclude that aging is accompanied by reduced expression of key enzymatic and non-enzymatic anti-oxidants in Leydig cells leading to excessive oxidative stress and enhanced oxidative damage (lipid peroxidation). It is postulated that such excessive oxidative insult may contribute to the observed age-related decline in testosterone secretion by testicular Leydig cells.

Keywords: Free radicals; Leydig cells; Lipid peroxidation; Steroidogenesis; Oxidative stress; Vitamin E; Ascorbic acid; Glutathione; Superoxide dismutases; Catalase; Glutathione peroxidase

1. Introduction

Aging in the testicular Leydig cells is accompanied by significant reduction in testosterone synthesis and secretion [1–7]. Although the mechanism behind this aging defect is not well understood, previous studies from this laboratory suggest that it may involve inefficient processing of intra-cellular stored cholesterol and/or transport of cholesterol to mitochondrial CYP11A1 sites for enzymatic conversion of cholesterol to pregnenolone and subsequently to androgen [4]. More recently completed studies from this laboratory [8] and others [9,10] suggest that functional expression of the two putative cholesterol proteins, steroidogenic acute regulatory protein (StAR) and peripheral type benzodiazepine receptors (PBR), is greatly reduced during aging.

Excessive oxygen free radical generation [11–15] leading to lipid peroxidation, oxidative stress and excessive damage of cellular macromolecules (protein, lipids, nucleic acids) [16–21] has been hypothesized to be a major contributor to the aging process. In particular, lipid peroxidation is now considered to be a major mechanism by which oxygen free radicals can cause tissue damage leading to impaired cellular function, alterations in the physico-chemical properties of cellular membranes, apoptosis, and reduced enzyme/protein activity [18,21], and accumulation of lipofuscin aging pigments [22]. Because free radical formation is a normal occurrence during cellular respiration [23,24]

Abbreviations: Cu, Zn-SOD, Cu, Zn-superoxide dismutase; Mn-SOD, Mn-superoxide dismutase; GPX-1, glutathione peroxidase-1; CAT, catalase

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and specialized functions such as steroidogenesis [25,26], mammalian cells are equipped with both enzymatic and non-enzymatic anti-oxidant defense systems to cope with oxygen free radicals. The former includes a variety of anti-oxidant enzymes including Cu, Zn-superoxide dismutase (Cu, Zn-SOD), Mn-superoxide dismutase (Mn-SOD), catalase (CAT), and glutathione peroxidases (GPXs) [12,27–29], where as the latter consists of small molecular weight compounds such as α-tocopherol, ascorbic acid, β-carotene, and reduced glutathione [12,27,30,31]. However, there is now growing evidence, stemming mostly from studies involving non-steroidogenic cell/tissue systems, that the activities of certain anti-oxidant enzymes decline with age exacerbating oxidative stress conditions [18,32,33].

The risk of oxidative damage from lipid peroxidation is especially high for steroid synthesizing tissues, because these tissues, in addition to oxidative phosphorylation, use molecular oxygen for steroid biosynthesis, and all interactions of the cytochrome P450 enzymes with their substrates (cholesterol and its metabolites) are additional sources of oxygen free radical generation [25,26]. Indeed, it has been shown that free radicals inhibit steroidogenesis by interfering with cholesterol transport to mitochondria and/or catalytic function of P450 enzymes [34–39]. Thus, there is likelihood that chronic oxidative stress may be an important mechanism for the age-related loss of steroidogenesis, but to date the role of oxidative stress/excessive free radical formation in Leydig cell aging has not been determined. The present study was initiated to determine if the age-related decline in rat Leydig cell steroidogenesis was associated with excessive oxidative stress and if this was secondary to the loss of functional expression of non-enzymatic and/or enzymatic anti-oxidant defense systems.

2. Materials and methods

2.1. Materials

[α-^{32}P] CTP (sp. act. 29.6 TBq mmol^{-1}; 800 Ci mmol^{-1}) was purchased from Perkin Elmer (Wellesley, MA). Various cDNA probes were obtained from the sources described earlier [32]. The following reagents were supplied by Sigma (St. Louis, MO) bovine liver catalase, bakers yeast glutathione reductase, bovine liver superoxide dismutase, milk xanthine oxidase, reduced and oxidized forms of glutathione, nitroblue tetrazolium, xanthine, α-tocopherol, ascorbic acid, β-nicotinamide adenine dinucleotide phosphate (NADPH), ADP, and thiobarbituric acid. All other reagents used were of analytical grade.

2.2. Animals

All experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the Department of Veterans Affairs Palo Alto Health Care System (VAPHCS). Male Sprague–Dawley rats were used for all studies. Young rats at 2-months of age were purchased from Bantin and Kingman (Fremont, CA) and, maintained in our animal facility and used at 5-months of age (young-mature). Likewise, 11–12-months-old virgin rats were purchased from the same vendor and allowed to age to 24-months (old) in our animal facility in a room reserved for raising aged animals. The animals were individually housed, fed ad libitum, and used for experiments 4 h after fasting. In general, two pooled testes obtained from the same animal were used per experiment.

2.3. Isolation and purification of testicular Leydig cells

Testicular interstitial cells containing 10–15% Leydig cells were isolated by collagenase digestion of decapsulated testes obtained from 5- and 24-month-old rats. Highly purified (>85%) Leydig cell preparations were obtained by subjecting interstitial cell suspensions to isoosmotic Percoll density gradient centrifugation as previously described [4].

2.4. Measurement of lipid peroxidation

Membrane lipid peroxidation, as a measure of oxidative damage to lipids, was assessed by colorimetric determination of thiobarbituric acid-reactive substances (TBARs) [40]. TBARS formation was determined under basal condition (endogenous) and in the presence of Fe^{3+}/ADP/NADPH (enzymatic) [41] and Fe^{2+}/ascorbate (non-enzymatic) [42] as pro-oxidants. In brief, total cellular membranes were prepared from isolated Leydig cells by homogenizing cells in 10 mM Tris–HCl–150 mM KCl, pH 7.4, and sedimenting at 100,000 × *g* for 60 min. The pelleted membrane fraction was resuspended in the homogenizing medium to a concentration of 0.5 mg protein ml^{-1}. Due to the low yield of purified cells, Leydig cell total membranes instead of microsomal fractions were used. Suitable aliquots of cell membranes (500 μg protein ml^{-1}) were incubated (final volume 0.5 ml) for 1 h at 37 °C in 5 mM Tris–HCl, pH 7.4–150 mM KCl alone (basal lipid peroxidation), in the presence of 20 μM $FeCl_3$, 2.0 mM ADP, and 1.0 mM NADPH (enzymatic lipid peroxidation), or 26 μM $FeSO_4$ and 130 μM ascorbic acid (non-enzymatic lipid peroxidation). Peroxidation was stopped by the rapid addition of 7.5 μl of 2% butylated hydroxytoluene in ethanol followed by 2.0 ml of TBA–TCA–HCl reagent (0.375% TBA, 15% TCA, and 0.25 N HCl). The samples were mixed thoroughly and placed in a boiling water bath for 15 min. After cooling, the tubes were centrifuged to sediment the precipitated material and the absorbance of the clear supernatant was read at 535 nm. Malondialdehyde (MDA) formation was quantified using a molar extinction coefficient of 1.49×10^5 M^{-1} cm^{-1} [40]. The intra- and inter-assay coefficients of variation were 3 and 6%, respectively.

2.5. Enzyme assay methods

For determination of catalytic activity of anti-oxidant enzymes, purified Leydig cell preparations were homogenized in buffer (50 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA), briefly sonicated, and centrifuged at 600 × *g* for 10 min; supernatant fractions were stored at −90 °C until assayed for various enzymatic activities and protein content. Total SOD activity was assayed according to Spitz and Oberley [43]. The amount of enzyme that resulted 50% inhibition of NBT reduction was defined as one unit. Mn-SOD activity was measured in the presence of 2.0 mM sodium cyanide, an inhibitor of Cu, Zn-SOD [32]. Cu, Zn-SOD activity was calculated from the total activity minus the activity observed in the presence of sodium cyanide. Catalase activity was determined according the procedure of Aebi [44] with modifications [45]. Specific activity is expressed as κ units mg^{-1} of protein. Selenium-dependent glutathione peroxidase-1 (GPX-1) activity was assayed according to Lawrence and Burk [46] using H_2O_2, reduced glutathione and NADPH. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH min^{-1} mg^{-1} protein. The intra-and inter-assay coefficients of variation were 4–5 and 5–7%, respectively.

2.6. Quantification of mRNA levels by RNAse protection assay (RPA)

Total RNA from various Leydig cell preparations was isolated by the guanidine–isothiocyanate–phenol–chloroform extraction procedure of Chomczynski and Sacchi [47]. The concentrations of Cu, Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase-1 mRNAs were determined using a 'sensitive' and quantitative RPA as described in detail earlier [32,48]. In brief, aliquots of total RNA (5–20 μg) or control tRNA (10 μg) were hybridized with freshly synthesized ^{32}P-labeled anti-sense riboprobes for Cu, Zn-SOD, Mn-SOD, CAT, glutathione peroxidase-1, or 18S ribosomal RNA transcripts and the RNA:RNA hybrids were digested with RNAse A and T1 [32,48]. The protected fragments were separated on 6% acrylamide–urea denaturing gels. After electrophoresis, gels were exposed to Kodak XAR-5 film at −70 °C with intensifying screens to visualize the bands. For strong signals, gels were usually exposed for 6–12 h and for weaker signals for up to 48 h. For quantification, the autoradiographs were subjected to scanning densitometry and numerical values for each protected band were normalized to the 18S ribosomal RNA signal.

2.7. Miscellaneous measurements

The cellular content of non-enzymatic anti-oxidants, Vitamin E (α-tocopherol), Vitamin C (ascorbic acid), total glutathione, oxidized glutathione, and reduced glutathione were determined as previously described [32]. The intra- and inter-assay coefficients of variation were in the range of 4–6 and 5–8%, respectively. The procedure of Markwell et al. [49] was used to quantify protein content of cellular extracts or isolated membranes.

2.8. Statistical analysis

The results are expressed as the mean±S.E. The data were analyzed by two-way analysis of variance (ANOVA). Subsequently, the Student's *t*-test was performed to determine the significance of the differences between the mean values obtained for young and aged rats. A P value of <0.05 or less was considered to be statistically significant. The program used for statistical analysis was GRAPH PAD PRISM™, version 2.0 (Graph Pad software, San Diego, CA).

3. Results

3.1. Basal and pro-oxidant-induced lipid peroxidation

The TBARS assay was used to measure peroxidative damage (lipid peroxidation) in the Leydig cell membrane preparations from young (5-month) and old (24-month) rats and the results are presented in Table 1. Basal levels of TBARS content were increased approximately 200–300% in Leydig cell membrane preparations from old rats as compared with membrane samples from young animals ($P < 0.0060$). The production of TBARS in old Leydig cell membranes during the 1 h incubation in the presence of $FeCl_3$/ADP/NADPH (enzymatic) or $FeSO_4$/ascorbate (non-enzymatic) was slightly higher, though these values did not achieve statistical significance.

3.2. Non-enzymatic and enzymatic anti-oxidants

We next examined whether the increased TBARS formation (lipid peroxidation) in Leydig cell membrane preparations from old animals was due to alterations in the levels of non-enzymatic and/or enzymatic anti-oxidants. Table 2 provides data on cellular levels of three major non-enzymatic anti-oxidants, ascorbic acid, α-tocopherol, and glutathione (reduced and oxidized) in young and old Leydig cells. The isolated Leydig cells from 24-month-old rats showed a marked reduction in the cellular levels of reduced

Table 1
Basal and pro-oxidant-induced lipid peroxidation in Leydig cell membrane preparations from young and old rats (TBARS, nmol MDA produced mg^{-1} protein h^{-1})

Pro-oxidant	Young	Old
Basal	1.788 ± 0.265	4.860 ± 0.692[a]
Fe^{3+}/ADP/NADPH	23.09 ± 5.160	31.01 ± 5.713
Fe^{2+}/ascorbate	22.64 ± 5.461	27.10 ± 6.301

Results are mean ± S.E. of separate experiments.
[a] $P \leq 0.0060$.

Table 2
Levels of non-enzymatic anti-oxidants in freshly isolated Leydig cells from young and old rats (nmol mg^{-1} protein ± S.E.)

Non-enzymatic anti-oxidants	Young	Old
α-Tocopherol (Vitamin E)	0.109 ± 0.019	0.119 ± 0.014
Ascorbic acid (Vitamin C)	22.33 ± 7.19	18.20 ± 3.83
Reduced glutathione (GSH)	16.88 ± 3.56	6.48 ± 0.90[a]
Oxidized glutathione (GSSG)	0.150 ± 0.050	0.138 ± 0.025

Results are mean ± S.E. of four separate experiments.
[a] Different from GSH in Leydig cells from old rats with a $P \leq 0.0299$.

glutathione. In contrast, cellular levels of two other potent, non-enzymatic Leydig cell anti-oxidants, ascorbic acid and α-tocopherol as well as the oxidized form of glutathione (GSSG) were unaffected by the aging process (Table 2).

Catalytic activity and mRNA levels of the anti-oxidant enzymes, Cu, Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase-1 were also determined in Leydig cells from young and old rats. Relatively higher specific activities were noted for Cu, Zn-SOD, and GPX-1 in Leydig cell homogenates from both young and old rats (Table 3). In contrast, only low levels of mitochondrial Mn-SOD and peroxisomal catalase activities were detected. With the exception of catalase, which showed no significant change with age, all three remaining anti-oxidant enzymes exhibited a substantial decline in catalytic activity in Leydig cell extracts obtained from the older animals.

To correlate changes in enzyme activities with the respective mRNA levels, we also quantified the steady-state mRNA levels of Cu, Zn-SOD, Mn-SOD, catalase, and GPX-1 using a sensitive RNAse protection assay as described earlier [32]. Fig. 1 shows an autoradiograph obtained from a typical RPA run showing protected mRNA fragments for Cu, Zn-SOD, Me-SOD, catalase, GPX-1, and 18S rRNA using total RNA preparations from Leydig cells from young rats. Signals for Cu, Zn-SOD, Mn-SOD, and GPX-1 protected fragments were drastically reduced by aging such that twice the amount of total RNA (20 μg) and longer exposure times were required to generate weak signals (data not shown). Quantitative data presented in Fig. 2 demonstrate that similar to the loss of catalytic activities, aging also leads to down regulation of mRNA levels encoding the Cu, Zn-SOD, Mn-SOD, and GPX-1 enzymes. No significant age-related changes in the Leydig cell catalase mRNA levels, however, were noted between two age groups.

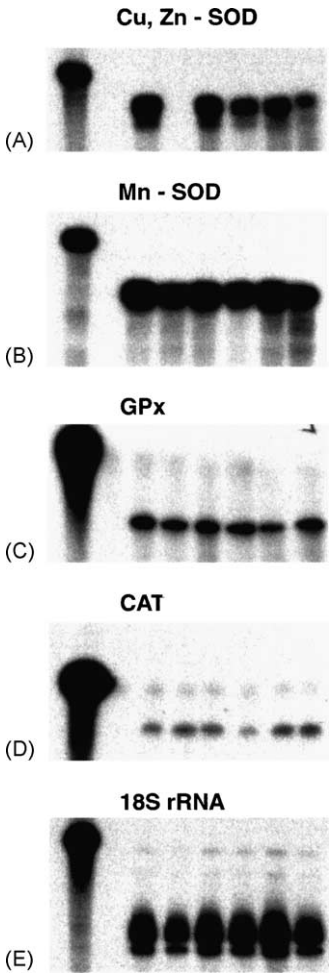

Fig. 1. Autoradiogram from a typical RNAse protection assay showing protected fragments for Cu, Zn-SOD, Mn-SOD, GPX-1, catalase, and 18S rRNA. Aliquots of total RNA from Leydig cells of young (Y) rats were hybridized with freshly prepared anti-sense cRNA probe specific for Cu, Zn-SOD, Mn-SOD, catalase, GPX-1, or 18S rRNA and the RNA:RNA hybrids were digested with RNAse A and T1. The protected fragments were resolved by electrophoresis followed by autoradiography.

Table 3
Effect of age on the anti-oxidant enzyme activities in rat Leydig cells (activity units mg^{-1} protein ± S.E.)

Enzymes	Young	Old
Cu, Zn-SOD	275.4 ± 62.1	117.4 ± 23.7[a]
Mn-SOD	42.4 ± 10.7	16.8 ± 2.9[b]
Catalase	14.4 ± 3.2	18.8 ± 4.8[c]
GPX-1	287.0 ± 51.0	154.0 ± 26.6[d]

Results are mean ± S.E. of five separate experiments.
[a] $P \leq 0.0447$.
[b] $P \leq 0.049$.
[c] $P \leq 0.4651$ (NS).
[d] $P \leq 0.0433$.

4. Discussion

The results presented here demonstrate that the age-related decline in testosterone secretion by testicular Leydig cells is accompanied by excessive oxidative stress and peroxidative cell damage. Enhanced oxidative stress in the aging Leydig cells is suggested by the significantly increased formation of TBARS formation (i.e. enhanced lipid peroxidation), especially under basal conditions. Because, aging in Leydig cells is also accompanied by selective loss of key non-enzymatic and enzymatic anti-oxidants, the breakdown of anti-oxidant defenses may directly contribute to

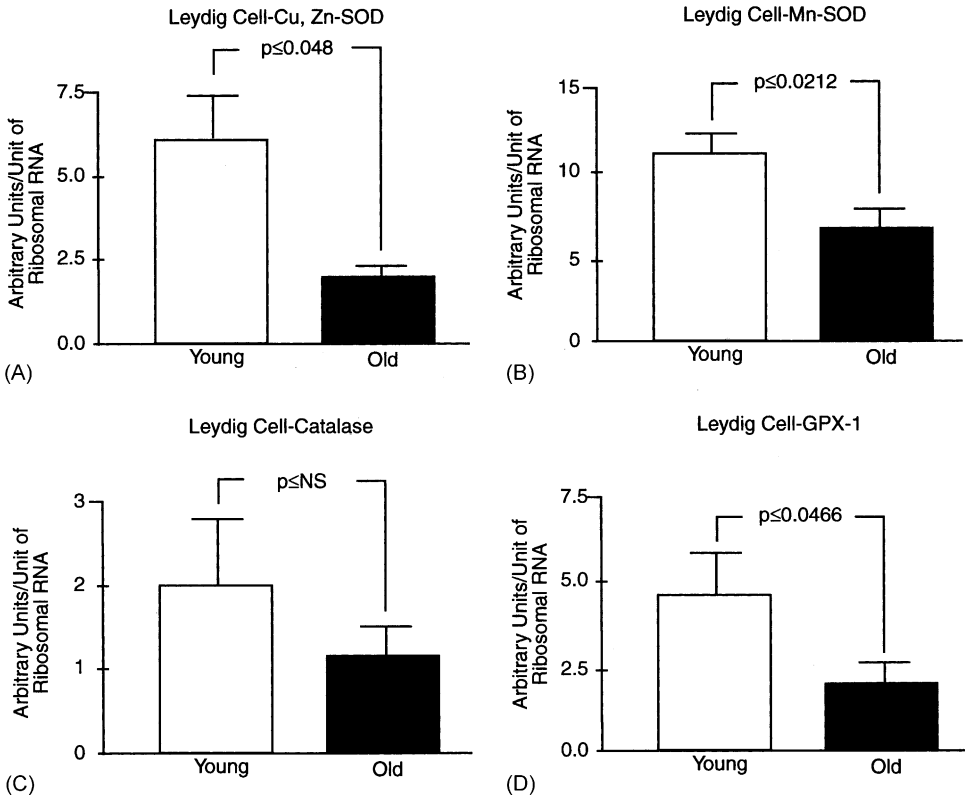

Fig. 2. Quantitative densitometry of RPA autoradiograms demonstrating that mRNA transcript levels of Cu, Zn-SOD, Mn-SOD, and GPX-1 are reduced in Leydig cells from aged rats. To evaluate the levels of Cu, Zn-SOD, Mn-SOD, GPX-1, catalase, and 18S rRNA, RPA were performed on total RNA extracted from Leydig cell preparations from 5-month (young) and 24-month (old) rats. Aliquots of 10 and 20 μg total RNA from young and old Leydig cells, respectively, were hybridized with anti-sense Cu, Zn-SOD, Mn-SOD, and GPX-1 cRNA probes. For catalase mRNA measurements, 20 μg aliquots of total RNA were used. To measure rRNA levels, 5 μg aliquots of total RNA were used in hybradization reactions. 18S rRNA was employed to normalize the scanning data. Data are representative of seven to nine separate measurements employing one rat per group.

oxidative stress, membrane lipid peroxidation and a decline in steroidogenic function. In mammalian cells, mitochondria are considered to be the major source of free radical formation as a by-product of oxidative phosphorylation [23,24]. Steroidogenic cell mitochondria generate additional free radicals as a product of steroidogenesis itself [25,26] and we speculate that increases in free radical formation relative to loss of anti-oxidant defense systems during aging render mitochondrial steroidogenic machinery and putative cholesterol transport proteins more susceptible to lipid peroxidation and oxidative damage leading to their functional inactivation.

Chen et al. [50] have attempted to quantify superoxide production as a measure of oxidative stress in Leydig cells isolated from the testis of old Brown Norway rats and concluded that aging cells generate significantly higher levels of superoxide radicals as compared with cells derived from young animals. However, these studies represented an indirect measure of oxidative stress since Leydig cells were incubated with a mitochondrial specific probe, lucigenin, and increased chemiluminescent signal generated upon interaction of probe with intra-mitochondrial superoxide was taken as a qualitative estimate of superoxide production. Also, from these studies, it was unclear what impact, if any, increased superoxide production had on the overall oxidative damage (lipid peroxidation) to cellular organelles. In the present studies, we have clearly shown that basal levels of lipid peroxidation were increased two- to three-fold in purified Leydig cells from old Sprague–Dawley rats. These data can be interpreted to suggest that aging-induced oxidative stress and increased free radical generation may cause excessive oxidative damage to cellular membranes and possibly affect the functional integrity and capacity of mitochondrial-associated steroidogenic machinery and/or accessory proteins that are involved in cholesterol transport to the mitochondria.

Our data also indicate that age-related increased free-radical generation and associated-oxidative damage is at least partially due to the functional imbalance of both enzymatic and non-enzymatic anti-oxidant defense systems. The activity and mRNA levels of Cu, Zn-SOD, Mn-SOD, and GPX-1 were significantly reduced in Leydig cell preparations from old rats. On the other hand, both the catalase catalytic activity and mRNA levels showed no changes with age. Among the key non-enzymatic anti-oxidants, only the levels of reduced glutathione (GSH) declined significantly in Leydig cells during aging. GSH participates in several major pathways involved in anti-oxidant defense including

reduction of toxic hydroperoxides catalyzed by glutathione peroxidases and some peroxiredoxins, and conjugation reactions catalyzed by glutathione-S-transferases [31]. It is possible that reduction in GSH could seriously impair the optimal functioning of these various catalytic activities, particularly the scavenging of superoxide radicals by glutathione peroxidases through the inter-mediary action of superoxide dismutases [12,27–29].

It is interesting to note that cellular levels of two other potent non-enzymatic anti-oxidants, α-tocopherol (Vitamin E) and ascorbic acid were not affected in response to old age. Vitamin E is the only lipid-soluble anti-oxidant embedded within biological membranes, clearly posses chain breaking anti-oxidant properties and is considered to be the most effective anti-oxidant weapon against lipid peroxidation [30,51]. However, in present studies no such association between Vitamin E and lipid peroxidation was noted, i.e. levels of α-tocopherol did not change in old Leydig cells compared with cells from young rats, yet significant differences were noted in the rates of lipid peroxidation. Like Vitamin E, the levels of ascorbic acid were also not affected during aging. Ascorbic acid is a water-soluble anti-oxidant, which exists, in high concentrations in many mammalian tissues including steroidogenic tissues like the adrenal gland [32]. It scavenges many different types of free radicals including superoxide, hydrogen peroxide, hydroxy radicals, singlet oxygen, and reactive nitrogen species [30] and is also capable of regenerating α-tocopherol from the tocopheroxy radicals that are generated during the α-tocopherol-mediated inhibition of lipid peroxidation [30]. The levels of both Vitamin E and ascorbic acid did not change with age, but at the same time the levels of GSH and anti-oxidant enzymes (which scavenge superoxide anion and convert them to harmless water) declined significantly, suggesting the possibility that these two non-enzymatic anti-oxidants share a greater burden for interrupting the propagation of polyunsaturated fatty acid oxidation and progression of lipid peroxidation. Obviously, additional future studies are necessary to evaluate the exact functional roles that anti-oxidants, particularly Vitamin E and ascorbic acid, play in the delay, prevention, and perhaps reversal of the age-related reduction in testosterone secretion by Leydig cells.

In conclusion, free-radical-induced lipid peroxidation and oxidative damage to cellular membranes is increased with age in testicular Leydig cells, and this increased oxidative insult, in part, results from decreased functional expression and activity of key components of the anti-oxidant defense system. These data suggest that during aging, loss of Leydig cell steroidogenic response is potentially mediated by the increase in free radical-induced lipid peroxidation and that increased lipid peroxidation negatively affects the normal functioning of membrane-associated steroid producing machinery and/or putative cholesterol transport proteins (e.g. StAR, PBR, etc.). Further studies are in progress to test these various possibilities.

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