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Novel Connections Between NADPH-Induced Lipid Peroxidation and Cytochrome P450 Inactivation, and Antioxidant and Enzyme Protective Properties of Estradiol in Gonadal Membranes

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This study uses microsomal membranes from rat testis tissue, including the cytochrome P450c17 (steroid 17α-monooxygenase/17α-hydroxyprogesterone aldolase, catalyzing the conversion of progesterone to androstenedione), to decipher the possible relation of NADPH-induced (no exogenous iron added) lipid peroxidation and cytochrome P450 inactivation and the protective effect of certain steroids. NADPH (300 μM) causes a 3.6-fold stimulation of malondialdehyde formation (thiobarbituric acid-reactive substances) and a 29% cytochrome P450c17 loss within 1 h at 37°C, but has no effect on lipid peroxidation in the presence of the iron chelator desferrioxamine. Hydrogen peroxide has only marginal effects. The antioxidant efficiency of estradiol (IC_{50} = 13.9 μM) is higher than its cytochrome P450c17 protective efficiency (IC_{50} = 33.0 μM), whereas androstenedione does not inhibit lipid peroxidation but protects cytochrome P450c17 completely. The human choriogonadotropin-induced degradation of cytochrome P450c17 in incubated decapsulated testes can not be correlated with a stimulation of lipid peroxidation, and it is partially inhibited by estradiol but completely abolished by androstenedione. It is concluded (I) that NADPH stimulates iron-dependent generation of reactive oxygen species by the monooxygenase system even in the presence of certain P450 ligands in the physiological membrane environment, (II) that membrane lipid peroxidation may be suppressed by hydrophobic steroids acting as antioxidants such as estradiol, (III) that steroid ligands stabilize cytochrome P450c17 against inactivation in the presence of NADPH even if they do not act as substrates and do not possess antioxidant activity, and (IV) that the choriogonadotropin-induced down-regulation of cytochrome P450c17 is not due to accumulating steroids acting as "pseudosubstrates" as occasionally supposed.

Keywords: Cytochrome P450, membrane lipid peroxidation, antioxidants, estrogens, androgens, gonadotropins

INTRODUCTION

Generation of reactive oxygen species, which predominantly arise as a consequence of abortive intermediate escape during enhanced oxygen activation reactions, marks a situation of so-called oxidative stress potentially leading to structural

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and functional alterations of macromolecules and supramolecular assemblies. One consequence is the peroxidation and partial breakdown of polyunsaturated lipids in membrane systems and lipoproteins. Cells contain a battery of enzymatic and non-enzymatic antioxidative defense systems which may primarily act either in the aqueous compartment or in hydro-phobic membrane phases.[1,2] Both endogenous and nutritive compounds with phenol or catechol moieties have repeatedly been described as chain-breaking antioxidants; amongst these, estrogens have been particularly well characterized because of their physiological (e.g., neuroprotective) importance.[3–6] However, there are additional reports confining the antioxidant significance of estrogens by showing that metabolism of these hormones can also result in opposite effects.[7,8]

Cytochrome P450-dependent mitochondrial and microsomal monooxygenation systems have been identified as one important source of reactive oxygen species.[9–13] Those systems localized in smooth endoplasmic reticulum membranes comprise two protein components, namely the flavoprotein NADPH:cytochrome reductase (EC 1.6.2.4), which oxidizes NADPH to form one pair each of protons and electrons, and a cytochrome P450 (EC 1.14), which activates and splits one oxygen molecule, usually yielding H_2O and an oxidatively modified product. Abortive leakage of incompletely processed oxygen species may result in oxidative damage of cytochrome P450 itself and of other proteins (making these targets more susceptible to proteolytic degradation), as well as in surrounding membrane lipid peroxidation.[9–11,14–16]

Several cytochromes P450 are involved in the conversion of steroid hormones. Thus, the formation of one testosterone or one estradiol molecule from cholesterol requires the cytochrome P450-catalyzed activation of five or eight dioxygens, respectively.[17] Cytochrome P450c17 (steroid 17α-monooxygenase/17α-hydroxyprogesterone aldolase; EC 1.14.99.9/4.1.2.30) is a microsomal bifunctional enzyme controlling the metabolic branchpoint from gestagens to either corticoids or androgens. This enzyme protein is regulated in a complex pattern; for instance, it is down-regulated upon stimulation of the gonads with lutropin or choriogonadotropin (hCG).[18,19] This event has been proposed to depend on increased substrate flux, oxygen consumption, and/or androgen product accumulation in the hormonally stimulated endocrine glands.[20–24] On the other hand, physiologically circulating gonadotropin levels have recently been found to maintain intratesticular antioxidative enzyme (catalase, glutathione peroxidase) activities in addition to their role in pursuing steroidogenic cytochrome P450-derived radical formation.[16]

In the present study, interrelations between testicular microsomal lipid peroxidation, cytochrome P450c17 inactivation, and antioxidative protection are investigated in the presence of NADPH, but without exogenously added iron ions, and the possible significance of these processes for hCG-induced cytochrome P450c17 degradation is examined.

METHODS

Chemicals, if not stated otherwise, were purchased from Merck, Darmstadt, or from Boehringer, Mannheim, Germany, and were of the highest quality available. Androstenedione and progesterone were from Calbiochem, Bad Soden, desferrioxamine mesylate was obtained from Sigma Chemie, Deisenhofen, and ketoconazole was a gift from Janssen, Neuss, Germany. Testes from young postpubertal (240g) Wistar rats were used throughout. Microsomal membranes were prepared from tissue homogenates and extensively washed [in order to remove cytosolic components such as catalase][25] exactly as described previously.[26] Final pellets were resuspended in 20 mM Tris, 250 mM sucrose (= TS; pH 7.4) and found to contain 890 pmol cytochrome P450c17 per testis [using ε = 91000 l/(mol*cm) for the CO-induced spectra, ε = 120000 l/(mol*cm) for the proges-

terone-induced (type I ligand-induced) spectra and ε = 78000 l/(mol*cm) for the ketoconazole-induced (type II ligand-induced) spectra] and 240 pmol cytochrome b5 per testis;[26,27] spectral analyses were made in a Shimadzu Seisakusho (Kyoto, Japan) UV300 spectrophotometer. Liposomes were prepared from testis or analogously obtained hepatic microsomes by chloroform/ methanol (2 + 1 v/v) extraction and ultrasonic resuspension of the evaporated extracts in TS.[26]

If generation of reactive oxygen species was induced with NADPH (concentrations specified in the figures), membranes (0.39 ml microsomes or liposomes corresponding to 0.2 testis equivalents/ml final assay volume) were incubated for up to 1 h at 37°C together with 0.6 ml of 50 mM phosphate buffer (pH 7.4) including 280 μM $MgCl_2$ [in order to simulate otherwise used metabolic conditions][28] and appropriate additions; steroids were added in 10 μl methanol, and controls received 10 μl methanol. Desferrioxamine as an iron-chelator was added in 10 μl amounts of water in order to probe potential effects of endogenous iron.[29] When reactive oxygen species formation was excited with a preformed complex comprising 0.1 mM $FeCl_3$ and 1.6 mM ADP (final concentrations) plus 0.5 mM ascorbate,[3] microsomal or liposomal membranes (0.01 testis equivalent or 0.0008 liver equivalent/ml final volume) were incubated up to 15 min at 37°C. In one experiment, an aqueous system (without membranes) was used consisting of 3.3 μM $FeCl_3$ and 3.4 μM EDTA plus 0.3 mM ascorbate, 0.1 mM H_2O_2 and 70 μM murexide[30] for assessment of antioxidant properties of estradiol (37°C, up to 20 min). To characterize possible consequences of a stimulation of testicular steroidogenesis, testes were decapsulated and incubated in 10 ml Minimum Essential Medium Eagle with Earle's salts (ICN, Meckenheim, Germany) for 2 h at 37°C without or with 1 mg/l human choriogonadotropin (hCG; Primogonyl; Schering, Berlin, Germany) and steroids as indicated in Figure 4. Homogenates and microsomes were prepared thereafter as described above.

Cytochrome P450c17 concentrations were quantified spectroscopically as mentioned above; at least two different titration methods were used to exclude possible (yet not confirmed) effects of endogenous ligands. Formation of malondialdehyde as one indicator of membrane lipid peroxidation was assayed after coupling to thiobarbituric acid and extraction with 1-butanol by absorption photometry at λ = 535 nm exactly as described.[31] Linearity of malondialdehyde accumulation with time was established within the above-mentioned incubation periods; for instance, liberation of thiobarbituric acid-reactive substances by testicular microsomes under basal conditions exceeded the zero control value by 16%, 39% and 53% after 20, 40 and 60 minutes, respectively, while corresponding values with 300 μM NADPH amounted to 37%, 126% and 195%, respectively (n = 2). Neither estradiol nor androstenedione (at 200 μM concentrations) interacted with the thiobarbituric acid test as proved by lack of influence when added after a 1-hour incubation in the presence of NADPH. The rate of murexide bleaching by active oxygen species was followed at λ = 520 nm and found to be linear for at least 20 min.[30] Estradiol concentration-effect relationships were linearized after logit [i.e., log (x/1-x)] transformation of the residual effects and log transformation of ligand concentrations.

RESULTS

Malondialdehyde formation from membrane lipids of rat testis microsomes is profoundly stimulated in the presence of NADPH (20 to 600 μM range) in a dose-dependent fashion even in the absence of exogenously added iron ions (Fig. 1A). There is only a marginal stimulation of lipid peroxidation upon addition of 300 μM NADPH in liposomal vesicles prepared from testis microsomes (by a factor 1.2 for identical lipid amounts; n = 4) when compared to the NADPH effect on microsomal membranes (by a factor 3.6; see Fig. 1A). If testis liposomes are subjected to the

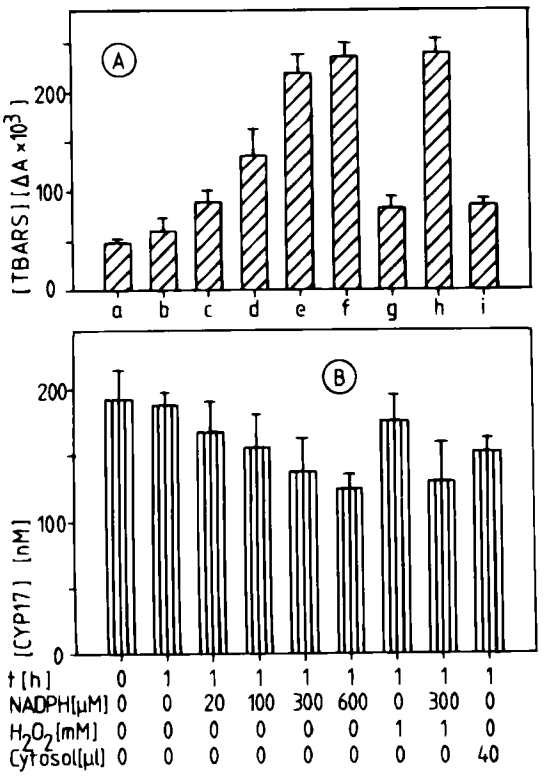

FIGURE 1 Effects of NADPH, H_2O_2 and testicular cytosol (0.01 testis equivalents/ml final assay volume) on malondialdehyde formation (measured at λ = 535 nm as thiobarbituric acid-reactive substances = TBARS, panel A) and cytochrome P450c17 concentrations (= CYP17, measured by progesterone-induced and CO-difference spectra; panel B) in isolated rat testis microsomes at 37°C (0.2 testis equivalents/ml). Means ± standard deviations (SD) from n = 4 to 6 independent preparations are presented; incubation details are found in the bottom lines.

$FeCl_3$/ADP/ascorbate system, however, a 28-fold (n = 7) enhancement of malondialdehyde release in comparison to iron-free controls occurs, indicating their potential susceptibility towards lipid peroxidation. Addition of 1 mM H_2O_2 without or with 300 μM NADPH induces only a slight acceleration of membrane lipid peroxidation as compared to the respective controls (Fig. 1A). These effects of NADPH and H_2O_2 are accompanied by a similarly concentration-dependent inactivation of testicular microsomal cytochrome P450c17 (Fig. 1B). Addition of a minute amount of testicular cytosol to microsomal membranes in the absence of NADPH and exogenous iron ions, which was originally undertaken to investigate potential antioxidant effects, rather results in a 1.7-fold increase in malondialdehyde formation and in a 18% loss of microsomal P450c17 (Fig. 1).

To consider a potential role of endogenous, membrane-bound iron ions, titrations with desferrioxamine (1:2 dilutions from 8 μM to 60 nM) were performed. In the presence of 300 μM NADPH, 2 μM desferrioxamine blocks malondialdehyde formation by testis microsomes completely, indicating a possible contribution of endogenous iron. No inhibition is obtained with 60 nM. Accordingly, 300 μM NADPH fails to produce any cytochrome P450c17 decay in the presence of 2 μM desferrioxamine. However, desferrioxamine at this concentration itself inactivated 16% of initial cytochrome P450 within 1 hour in the absence of NADPH.

There is a concentration-dependent counteracting effect of estradiol with respect to NADPH-induced lipid peroxidation and cytochrome P450c17 degradation in testicular microsomes; with sufficiently high hormone concentrations (200 μM), the protection of the membrane system is almost complete (Fig. 2). A detailed analysis of the estradiol concentration-effect relationship based on the respective NADPH-induced effect as the 100% reference value reveals a higher sensitivity of lipid peroxidation (50% protecting estradiol concentration: IC_{50} = 13.9 μM) than of P450c17 degradation (IC_{50} = 33.0 μM) towards the beneficial estradiol action (Fig. 3). It has been established in previous experiments[26] that estradiol binds to P450c17 and inhibits progesterone metabolism competitively with K_I = 23.5 μM. These values are in the same order of magnitude as corresponding measures for the antioxidant efficiency of estradiol in other systems: Its IC_{50} for inhibition of murexide bleaching in an aqueous, membrane-independent system comprising $FeCl_3$/ADP/ascorbate/H_2O_2 amounts to 32.5 μM (n = 16), and the IC_{50} for inhibition of $FeCl_3$/ADP/ascorbate-stimulated malondialdehyde production by liposomes generated from rat liver microsomal membranes amounts to 28.1 μM and

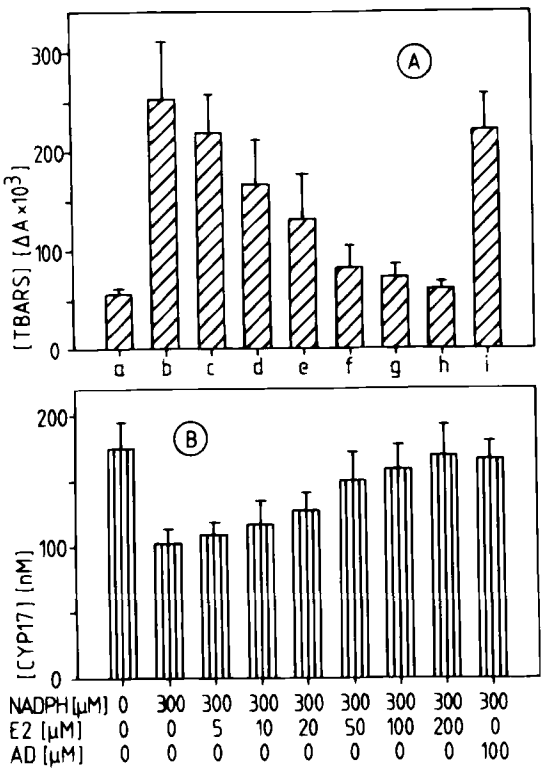

FIGURE 2 Effects of estradiol (= E2) or androstenedione (= AD) on formation of thiobarbituric acid-reactive substances (panel A) and cytochrome P450c17 concentrations (panel B) in isolated rat testis microsomes within 1 h at 37°C. Means ± SD from n = 6 independent preparations are given; see bottom lines and legend to Figure 1 for further details.

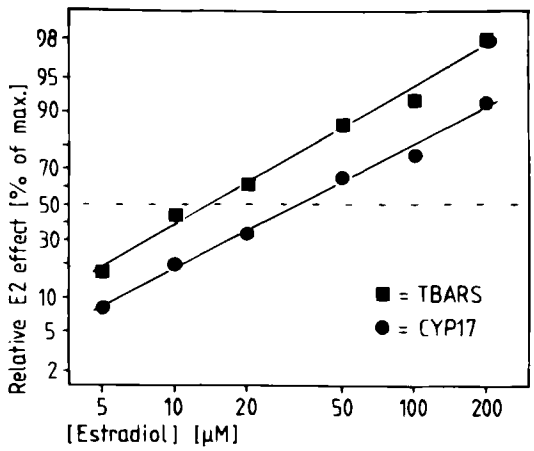

FIGURE 3 Replot (logit-log transformation) of the estradiol concentration versus effect relationship from the experimental data in Figure 2, columns c to h. The maximal effect (100% reference) is the difference between columns a and b in Figure 2, panels A or B, respectively.

14.8 μM estradiol in the presence of 150 μg and 50 μg lipid/ml final assay volume, respectively (data not shown).

Addition of 100 μM androstenedione, which is also a ligand for cytochrome P450c17[32], does not alter the rate of malondialdehyde formation from testis microsomes with 300 μM NADPH; yet, the NADPH-induced P450c17 degradation (58 nM/h) is markedly attenuated (17 nM/h) in the presence of androstenedione. This steroid is ineffective (up to 100 μM) in all the other systems tested for generation of reactive oxygen species. This indicates the possibility of an uncoupling between membrane lipid peroxidation and P450 inactivation under certain conditions at least in the experimental system employed here. Cytochrome P450c17 is likewise protected against NADPH-induced (600 μM) inactivation when performing maximal catalytic turnover of its physiological substrate, progesterone (10 μM added every 10 min): After 1 h, 144 ± 20 nM (x ± SD; n = 6) P450c17 remains in the presence of NADPH alone, whereas 178 ± 29 nM are recovered ($P < 0.05$) with NADPH plus progesterone, the P450c17 control level (no incubation) amounting to 184 ± 10 nM in this experimental series (n = 5).

The final experiment tests the possible involvement of lipid peroxidation in the hormonal regulation of rat testicular cytochrome P450c17. If decapsulated testes are incubated for 2 h in the presence of a maximally stimulating concentration of hCG (4.7-fold stimulation of testosterone secretion as compared to controls), only a slightly higher (9%; statistically insignificant) accumulation of malondialdehyde in tissue homogenates is found, which is not altered in the presence of 200 μM estradiol or 100 μM androstenedione. Yet, a 48% diminution of microsomal P450c17 levels occurs in the presence of hCG. The protective effect of 200 μM estradiol under these conditions is much less pronounced than expected on the basis of the membrane experiments, whereas this hCG

effect is completely abolished by androstenedione (Fig. 4). This is an indication that steroid accumulation and lipid peroxidation do not synergistically account for gonadotropin-induced P450c17 down-regulation in gonadal membranes.

DISCUSSION

Estrogens as well as numerous other natural compounds with phenol or catechol moieties have repeatedly been described as effective antioxidants.[3,5,33] Their membrane interaction properties and hydrophobicities are obviously crucial determinants of local action efficiencies.[26,33] In most cases, this feature has been recorded in systems where generation of reactive oxygen species was initiated by addition of free or chelated iron ions,[3,5,34] but that experimental approach simulates the physiological situation (when transition metal ions are predominantly tightly protein-bound or complexed with negatively charged phospholipid head groups)[11,35] only arbitrarily[11] and does not allow a differentiation between membrane stabilizing[4] and redox equilibrium modulating actions[5] of potential antioxidants such as estrogens. Therefore, the present study investigates possible modifications of lipid peroxidation induced by NADPH–stimulation of

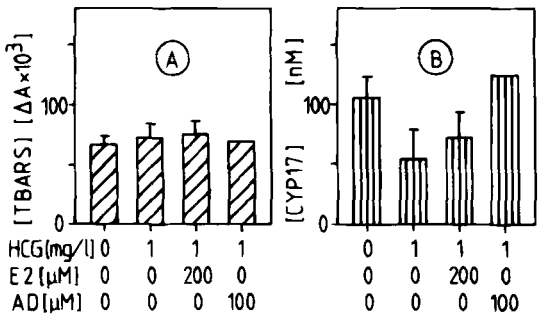

FIGURE 4 Modification by estradiol (=E2) or androstenedione (=AD) of hCG effects on formation of thiobarbituric acid-reactive substances (panel A) in homogenates and cytochrome P450c17 concentrations (panel B) in microsomes prepared from decapsulated testes incubated in medium for 2 h at 37°C. Means ± SD from n = 5 (n = 2 for AD) preparations (0.2 testis equivalents/ml) are given.

a membrane-associated monooxygenase system, but also considers potential effects of endogenous iron.[29] Testicular microsomes are employed since they contain one predominating and well-characterized[17,19,28] cytochrome P450 species (in contrast to P450 heterogeneity in liver microsomes), and since a specific role of lipid peroxidation in the regulation of this enzyme had still to be identified.[19] Further, antioxidant systems in the testis, especially in its endocrine compartment, are of particular importance[16,36] since the conversion of cholesterol to steroid hormones comprises a sequence of multiple oxygen activation reactions by membrane-bound P450s[17] including the possibility of abortive leakage of labilized, reactive oxygen species which may eventually attack macromolecules and intracellular structures.[11,14,15]

As expected, addition of NADPH to the microsomal cytochrome P450c17 system results in malondialdehyde accumulation and cytochrome P450 labilization. The control experiments reveal that this lipid peroxidation is at least partly due to endogenous iron ions. The lack of an NADPH-dependent effect on liposomes may be explained by removal of iron during the lipid extraction process employing organic solvents and/or by the loss of cytochrome P450 and NADPH: cytochrome reductase which otherwise provide electrons for oxygen reduction.[9,11,37] Accordingly, it has previously been suggested that full reduction of the NADPH: cytochrome reductase does not initiate membrane lipid peroxidation in the absence of exogenous iron or a P450, but obviously requires the (heme-)iron as the partner for oxygen activation.[9,29,37] On the other hand, several P450 isoforms can be inactivated by definite lipid peroxidation products in isolated systems in the absence of reductase.[38] The observation that hydrogen peroxide, which can arise from cytochrome P450-catalyzed abortive oxygen activation,[15] exerts only moderate effects in the present system (Fig. 1) in contrast to reconstituted hepatic isozymes,[39] is an indication that testicular cytochrome P450c17 inactivation may be a conse-

quence of membrane lipid modification rather than of direct oxygen-induced protein damage. This conclusion is in line with recent findings that P450c17 function is extremely sensitive towards variations in membrane lipid composition.[40] The slight acceleration (rather than inhibition) of malondialdehyde formation and P450c17 decay by testicular cytosol may be a consequence of supply of additional iron ions and may be seen in the context of the surprisingly low levels of testicular (as compared to hepatic) antioxidative defense systems.[41]

The present study proves that estradiol is a protective agent with respect to both membrane lipid and cytochrome P450 structures, though its primary site of action can at first not exactly be identified: Estradiol is a ligand of cytochrome P450c17 and may thus inhibit oxygen activation;[9,26,28] and it is also a chain-breaking reagent that accumulates to a high degree (partition coefficient Kp = 310) in microsomal membrane lipids.[3,26] However, a differentiation becomes possible upon comparison with the action of androstenedione which does not act as an inactivator of reactive oxygen species in accordance with previous results.[3] Furthermore, it does obviously not reduce generation of reactive oxygen by the monooxygenase system. According to the results presented herewith (Fig. 2), both estradiol and androstenedione presumably allow oxygen binding and activation after their accomodation by cytochrome P450c17, but do not act as oxidizable substrates (testosterone is an inappropriate ligand here since it can be oxidized to androstenedione by P450c17).[42] Therefore, activated oxygen species may leave the P450c17 active site[16] and become effective in membrane lipid damage in the presence of androstenedione but not with estradiol. On the other hand, it is important to note that steroid ligands, whether substrates (progesterone) or not, protect P450c17 effectively against inactivation even under conditions of still enhanced lipid peroxidation. This is probably achieved by a stabilization of protein structural domains and may be interpreted as a potential mechanism of partial "self-protection" of this enzyme under circumstances of increased substrate availability, catalytic activity and product accumulation as they occur after hormonal stimulation of the testes.

It has repeatedly been argued that the gonadotropin-induced down-regulation of cytochrome P450c17 in ovaries and testes[18] may depend on cytochrome P450-mediated, androgen-supported abortive oxygen activation.[20–22] The failure to detect malondialdehyde as a lipid peroxidation product after hCG stimulation, in connection with the discovery of stabilizing (rather than labilizing) actions of androgens and estrogens, support the conclusion that such a mechanism, which was deduced from mouse Leydig cell culture experiments, does not prevail in the decapsulated and still fully hCG-responsive rat testis. Whereas 100 μM androstenedione and 200 μM estradiol were equally protective with respect to P450c17 in the NADPH-supported isolated microsomal membrane system, the former steroid turned out to be much more effective in testicular tissue, further supporting the assumption that different mechanisms account for hCG-induced and lipid peroxidation-induced P450c17 inactivation.

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