

## ANTIOXIDANT PROTECTIVE MECHANISMS AGAINST REACTIVE OXYGEN SPECIES (ROS) GENERATED BY MITOCHONDRIAL P450 SYSTEMS IN STEROIDOGENIC CELLS

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*Mitochondrial P450 type enzymes catalyze central steps in steroid biosynthesis, including cholesterol conversion to pregnenolone, 11 $\beta$  and 18 hydroxylation in glucocorticoid and mineralocorticoid synthesis, C-27 hydroxylation of bile acids, and 1 $\alpha$  and 24 hydroxylation of 25-OH-vitamin D. These monooxygenase reactions depend on electron transfer from NADPH via FAD adrenodoxin reductase and 2Fe-2S adrenodoxin. These systems can function as a futile NADPH oxidase, oxidizing NADPH in absence of substrate, and leak electrons via adrenodoxin and P450 to O<sub>2</sub>, producing superoxide and other reactive oxygen species (ROS). The degree of uncoupling depends on the P450 and steroid substrate. Studies with purified proteins and overexpression in cultured cells show consistently that adrenodoxin, but not reductase, is responsible for ROS production that can lead to apoptosis. In the ovary and corpus luteum, antioxidant enzyme activities superoxide dismutase, catalase, and glutathione peroxidase parallel steroidogenesis. Antioxidant  $\beta$ -carotene,  $\alpha$ -tocopherol, and ascorbate can protect against oxidative damages of P450 systems. In testis Leydig cells, steroidogenesis is associated with aging of the steroidogenic capacity.*

*Key Words:* Ascorbate; Adrenodoxin; Superoxide; Catalase; P450<sub>sc</sub>; Tocopherol; Vitamin C; Vitamin E.

### Electron Transfer in Mitochondrial P450 Systems

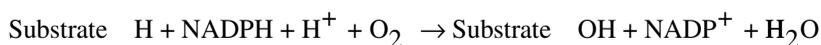
Cytochrome P450 type enzymes represent the largest superfamily of enzymes that are involved in the metabolism of generally small hydrophobic molecules. These enzymes share a common structural topology with a heme group in their active site and derive their name from the absorption maximum of their reduced carbon monoxide complex at 450 nm (Coon, 2005; Estabrook, 2005). In eukaryotes there are two classes of P450s that are located in the mitochondria and the endoplasmic reticulum. The nomenclature for P450 genes starts with the root name CYP and follows the order CYP:family:subfamily:gene (Nelson et al., 1996).

Mitochondrial P450 type enzymes are generally involved in the biosynthesis of cholesterol derived steroidal compounds (Hanukoglu, 1992; Miller, 2005). In mammals, the reactions catalyzed by these enzymes include cholesterol conversion to pregnenolone

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(which is the first step in steroid hormone biosynthesis in all steroidogenic tissues), 11 $\beta$  and 18 hydroxylation reactions in glucocorticoid and mineralocorticoid biosynthesis, C-27 hydroxylation of cholic acid in bile acid metabolism, and 1 $\alpha$  and 24 hydroxylations of 25-OH-vitamin D (Table 1). Sequencing of the drosophila genome has revealed several P450s with strong homology to animal mitochondrial P450s (e.g., Uniprot ID: C12B1\_DROAC). Yet, the biochemical functions of these enzymes in insects remain to be determined.

Hydroxylation reactions catalyzed by the mitochondrial P450s depend on NADPH as a source of reducing equivalents and molecular oxygen (O<sub>2</sub>). These P450s function as a monooxygenase catalyzing incorporation of one atom of O<sub>2</sub> into the substrate while the second atom of O<sub>2</sub> is reduced to water with the following stoichiometry:



In these reactions NADPH donates two electrons which are transferred to P450 via two electron transfer proteins, adrenodoxin reductase, which is an FAD containing flavoenzyme, and adrenodoxin, which is a [2Fe-2S] ferredoxin type iron-sulfur protein (Grinberg et al., 2000; Ziegler et al., 1999). FAD of adrenodoxin reductase accepts two electrons from NADPH, and these are transferred one at a time to adrenodoxin which is a one electron carrier. Thus, electrons are transferred in the following order:



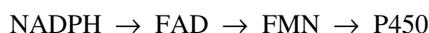
This order of electron transfer is similar to that of some bacterial P450 systems, such as P450cam from *Pseudomonas putida*, that includes a ferredoxin reductase and a ferredoxin (named putidaredoxin) as the electron transfer proteins (Schiffler and Bernhardt, 2003). In contrast, the microsomal P450 systems are dependent on a different type of reductase with two coenzymes, FAD and FMN (Degtyarenko and Kulikova, 2001). FAD can be reduced by two electrons. FMN can accept only one electron at a time from the

**Table 1** Reactions catalyzed by animal mitochondrial P450 systems.

P450	Gene	Uniprot	Major reaction	EC	Highest levels in ID
P450scc	CYP11A1	CP11A	Cholesterol side chain cleavage	1.14.15.6	Steroidogenic cells in adrenal cortex and gonads
P450c11	CYP11B1	C11B1	Steroid 11 $\beta$ hydroxylation	1.14.15.4	Zona fasciculata of adrenal cortex
P450c18	CYP11B2	C11B2	Steroid C-18 hydroxylation	1.14.15.5	Zona glomerulosa of adrenal cortex
P450cc24	CYP24A1	CP24A	25-OH-vitamin D3-24 hydroxylation	–	Kidney tubules
P450c27	CYP27A1	CP27A	Sterol C-27 hydroxylation Vitamin D3-25 hydroxylation	–	Liver
P450c1 $\alpha$	CYP27B1	CP27B	25-OH-vitamin D3-1 $\alpha$ hydroxylation	–	Kidney

References for the individual sequences can be retrieved from the Uniprot database by searching for Uniprot ID + asterisk (\*) (e.g., CP11A\*) in the Uniprot ID/AC field.

FAD and then transfers it to the P450. Thus, in the microsomal P450 systems the order of electron transfer is:



All three proteins of the mitochondrial P450 systems are located on the matrix side of the inner mitochondrial membrane (Hanukoglu et al., 1990b). Whereas, the mitochondrial P450s behave as integral membrane proteins, the electron transfer proteins are soluble in the matrix (Hanukoglu et al., 1981a, 1981b). All three proteins are encoded as larger precursors, and their signal peptides are cleaved during transfer into mitochondria (Omura, 1998). In contrast to the multiplicity of P450 forms, there is one form of adrenodoxin reductase and adrenodoxin, each encoded by one or two similar nuclear genes in all animal species (Grinberg et al., 2000; Hanukoglu et al., 1987; Miller, 2005). Thus, the electron transfer proteins are not specific to individual P450s and serve as electron donors for different cytochromes P450 in different tissues. Adrenodoxin reductase and adrenodoxin are expressed in all human tissues examined (Brentano et al., 1992). Their highest levels of expression are observed in steroidogenic cells, especially in adrenal cortex and ovarian corpus luteum (Brentano et al., 1992; Hanukoglu and Hanukoglu, 1986). The levels of these proteins show no significant sex or inter-individual variation in bovine adrenal cortex (Hanukoglu and Hanukoglu, 1986).

Previous studies established several general principles of function for P450 system electron transport chains: 1) The reductases are generally expressed at much lower levels than P450s, there being only one molecule of reductase per about 10 or more molecules of P450 (Hanukoglu and Hanukoglu, 1986); 2) The protein components are independently mobile and do not form static multi-component complexes; 3) Proteins that are redox partners form transient high affinity 1:1 complexes during their random diffusions, in accordance with the principles of mass action. Dissociation constants of these protein-protein complexes are strongly influenced by the redox states of the proteins and other molecules in the environment, such as P450 substrate, ions, and phospholipids; 4) The transfer of an electron between two redox partners depends on the formation of a specific high affinity 1:1 complex between the two proteins (Hanukoglu and Jefcoate, 1980; Lambeth et al., 1982).

The process of electron transfer during enzyme activity may be "coupled" or "leaky." In a coupled P450 system all electrons from NADPH are utilized in substrate hydroxylation reactions. When 100% of the electrons from NADPH are used in monooxygenation, then the efficiency of the system is also 100%. If a portion of the electrons are transferred to other acceptors, such as O<sub>2</sub> leading to the production of oxygen radicals, then this process is referred to as "uncoupling" of electron transfer from hydroxylation, as "leaky" electron transfer, and as "futile" oxidation. Some of the microsomal cytochromes P450 are highly uncoupled (Ding et al., 1991; Kohno et al., 2005; Puntarulo and Cederbaum, 1996). The leakage of electrons from mitochondrial respiratory electron transfer chain is considered a major source of oxygen radicals in cells (Genova et al., 2003; Turrens, 2003; Vinogradov and Grivennikova, 2005). A recent study indicates that a low percentage of only 0.15% of electron flow is directed to H<sub>2</sub>O<sub>2</sub> formation in mitochondrial electron transport chain (St-Pierre et al., 2002). Yet, even this low rate may be sufficient to cause accumulation of oxidative damage, ultimately resulting in aging (St-Pierre et al., 2002).

In steroidogenic tissues, the concentrations of the mitochondrial P450 system components are as much as 10 times higher than other electron transfer chain enzymes, and microsomal P450s (Hanukoglu and Hanukoglu, 1986). Thus, even if these P450 systems

leak electrons at a low rate, their total capacity for free radical generation could be high, especially in steroidogenic tissues.

The following sections present a review of studies on the involvement of the mitochondrial P450 systems in the generation of reactive oxygen species (ROS), and on the antioxidant mechanisms that are involved in protecting against these free radicals.

### Studies with Purified Enzymes

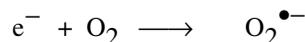
Within a cell with multiple factors it is difficult to examine the reactions of the individual components of a P450 system. Reconstituted systems with purified mitochondrial P450 system proteins have allowed examination of the reactions of each of the components of the system. The concentrations of NADPH, adrenodoxin reductase, adrenodoxin, and P450<sub>sc</sub> can be determined based on their extinction coefficients ( $\text{mM}^{-1} \times \text{cm}^{-1}$ ) at 340 nm (6.2), 450 nm (10.9), 414 nm (11), and 390 nm (110), respectively. Reduction or oxidation of these components can also be monitored using a spectrophotometer at a wavelength appropriate for each. The formation of the steroid product can be assayed using a specific assay. In a tightly coupled P450 system, the rate of NADPH oxidation would be expected to match the rate of substrate hydroxylation. In contrast, in a leaky system, a significant gap would be observed between these two parameters:

Coupled system:  $[\text{NADPH oxidized}] = [\text{substrate hydroxylated}]$

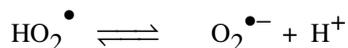
Leaky system:  $[\text{NADPH oxidized}] > [\text{substrate hydroxylated}]$

If reaction kinetics show a leaky system, the next step is then to identify the molecule that is accepting the electrons. Experiments using purified mitochondrial P450 system proteins showed that these systems can oxidize NADPH in the absence of a steroid substrate (Hanukoglu et al., 1993; Rapoport et al., 1995). Depletion of  $\text{O}_2$  in solution using glucose oxidase revealed that  $\text{O}_2$  is the acceptor of the leaking electrons (Hanukoglu et al., 1993). ESR assays using a spin trap and other assays provided further evidence that leaking electrons react with  $\text{O}_2$  to produce superoxide radical (Hanukoglu et al., 1993).

**Basic reactions and assay of superoxide radical.** Transfer of a single electron to  $\text{O}_2$  results in the formation of a superoxide radical.

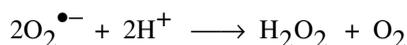


Superoxide radical is a highly reactive molecule that can participate in a series of reactions. Superoxide is the base form of a weak acid, the hydroperoxyl radical,  $\text{HO}_2^{\bullet}$



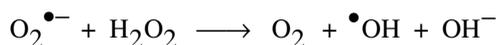
The pK of this reaction is 4.8. Thus, at  $\text{pH} = 7$  only about 1% of superoxide molecules are in the hydroperoxyl form.

Two superoxide radicals can react with two protons to produce hydrogen peroxide. This reaction is called a “dismutation”:

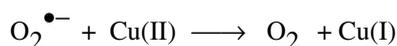
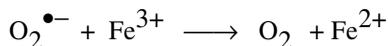


The same reaction is catalyzed by the enzyme superoxide dismutase (SOD), which has one of the highest turnover numbers among enzymes. Hydrogen peroxide can also be reduced to water by glutathione peroxidase (Fig. 1).

Superoxide can react with hydrogen peroxide to produce highly reactive hydroxyl radical (Haber-Weiss reaction):

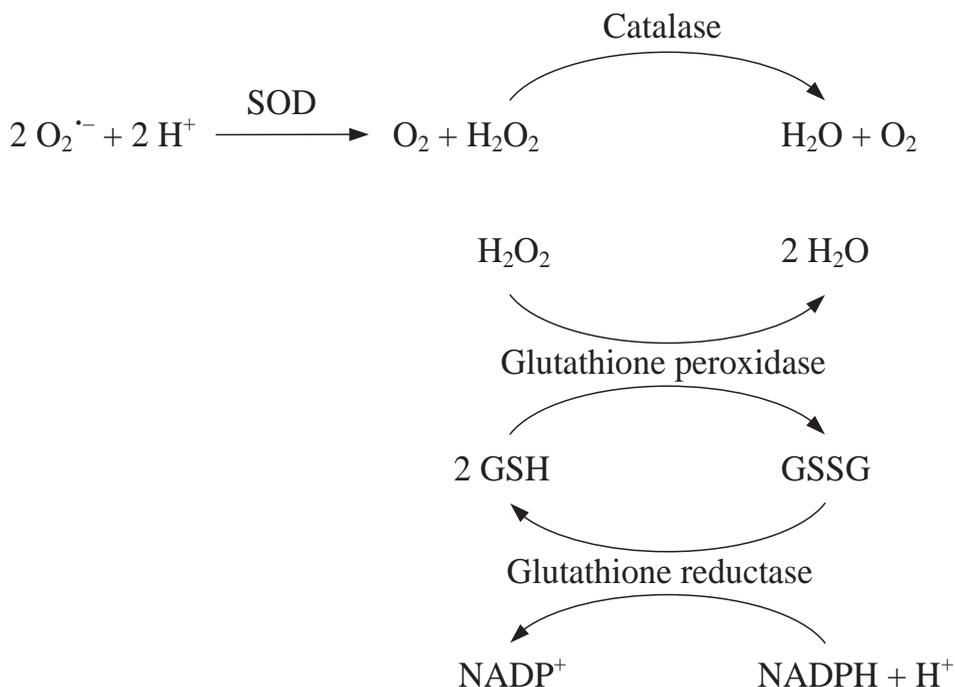


Superoxide can reduce ionic forms of iron and copper:



In addition to these basic reactions, oxygen radicals may also react with macromolecules such as lipids, sugars, proteins, and nucleic acids. The chemistry of these complex oxidation reactions has been reviewed (Cooke et al., 2003; Inoue et al., 2003; Marnett et al., 2003; Stadtman, 2001).

The assay of superoxide radicals is difficult because of their high reactivity and short half-lives. Commonly used assays are based on the spectrophotometric measurement



**Figure 1** The activities of antioxidant enzymes. Superoxide dismutase (SOD) catalyzes direct dismutation of superoxide to H<sub>2</sub>O<sub>2</sub>. Catalase catalyzes decomposition of hydrogen peroxide into oxygen and water without the production of free radicals. Glutathione peroxidases catalyze reduction of peroxides using glutathione (GSH) as a source of reducing equivalents. The product of the reaction, oxidized glutathione (GSSG), is recycled to its reduced state (GSH) by the enzyme glutathione reductase that uses NADPH as a source of reducing equivalents.

of products that are produced after reaction with superoxide radical; e.g., nitroblue tetrazolium reduction to monoformazan, epinephrine oxidation to adrenochrome, and cytochrome c reduction (Tarpey et al., 2004). These methods are relatively simple, but because of frequent nonspecific color reactions most cannot be used in quantitative assays. Some of these molecules, such as nitroblue tetrazolium and cytochrome c, are not suitable for study of enzymatic electron transfer systems that can reduce the molecule directly rather than through oxy-radicals. Oxy-radicals can be assayed using spin-traps with ESR spectroscopic equipment (Buettner and Mason, 1990). This method is difficult to use for quantitative kinetic assays because the rates of reaction of the spin-trap molecule with oxygen radicals often do not reflect the rate of radical formation. In reconstituted systems with purified enzymes, superoxide has been quantitatively assayed after dismutation to  $H_2O_2$  by superoxide dismutase (Rapoport et al., 1994). In contrast to superoxide,  $H_2O_2$  is relatively stable and can be assayed by  $H_2O_2$  dependent peroxidation or hydroxylation of compounds that can be measured by absorbance or fluorescence (Rapoport et al., 1994). Fluorimetric methods are generally more sensitive; however, these methods cannot be used in reactions with pyridine nucleotides, such as NADPH, because of their strong overlapping fluorescence.

Intracellular production of superoxide has been measured using dihydroethidium, a cell-permeant compound that can undergo a two-electron oxidation to form ethidium bromide, which can then be measured by flow cytometry of cells in suspension using fluorescence-activated cell sorter (FACS) (Tarpey et al., 2004). Another approach for superoxide detection employs chemiluminescent probes, such as lucigenin, that emit light during reaction with superoxide radicals. The low intensity signal of these probes require sensitive luminometers. The problems of sensitivity and specificity of these methods have been discussed in recent reviews (Brandes and Janiszewski, 2005; Tarpey et al., 2004).

**Electron leakage from adrenodoxin reductase and adrenodoxin.** Adrenodoxin reductase (AdR) is an FAD-containing enzyme that has two substrates, NADPH and adrenodoxin. AdR oxidizes NADPH, accepting two electrons. AdR then reduces adrenodoxin by transferring a single electron at a time (Chu and Kimura, 1973; Lambeth and Kamin, 1976). Adrenodoxin reductase is specific for NADPH as its  $K_m$  for NADPH and NADH are 1.8  $\mu$ M and 5.56 mM, respectively (Chu and Kimura, 1973). The sequence of AdR revealed a specific  $NADP^+$  binding motif that is present in many unrelated NADPH dependent enzymes (Hanukoglu and Gutfinger, 1989). Crystal structure of AdR confirmed the importance of this motif in determining the NADPH versus NADH specificity of the enzyme (Ziegler et al., 1999).

The ability of AdR and adrenodoxin to transfer electrons to  $O_2$  is strongly dependent on the formation of complexes between these proteins. In aerobic solution, adrenodoxin reductase that is reduced by NADPH remains reduced and does not readily oxidize by itself (Chu and Kimura, 1973; Hanukoglu et al., 1993; Sugiyama et al., 1979). Addition of adrenodoxin greatly stimulates the rate of NADPH oxidation and  $O_2$  reduction. Thus, reductase-adrenodoxin couple functions as a strong futile NADPH oxidase (Hanukoglu et al., 1993). The rates of electron leakage in the presence of different combinations of P450 system components are summarized in Table 2.

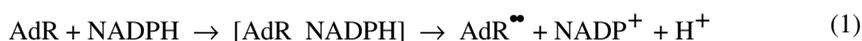
Kinetic events leading to the formation of superoxide radical can be summarized in four steps. In the following reactions, a dot ( $\cdot$ ) represents a single electron and the square brackets, [], signify an intermediate complex.

**Table 2** The rates of electron transfer from NADPH to O<sub>2</sub> or cytochrome c by the bovine mitochondrial P450 system components.

Reactants	Electron acceptor	Rate (mol e <sup>-</sup> /min/mol AR)	V <sub>max</sub> (μmol e <sup>-</sup> /min)
Adrenodoxin reductase (AdR)	O <sub>2</sub>	<0.001	
AdR + adrenodoxin	O <sub>2</sub>	149 ± 34	3.5 ± 0.3
AdR + adrenodoxin + P450scc	O <sub>2</sub>	721 ± 125	7.8 ± 2.1
AdR + adrenodoxin	Cytochrome c	387 ± 19	

All the rates are based on aerobic reactions carried out at 37°C, in 10 mM Hepes, pH 7.2, and 100 mM KCl (Hanukoglu et al., 1993). Cytochrome c is not a natural acceptor of electrons from adrenodoxin.

In the first step, AdR oxidizes NADPH by transferring two electrons to the FAD coenzyme:



In the second step, reduced AdR (AdR<sup>••</sup>) reduces adrenodoxin by transferring a single electron:

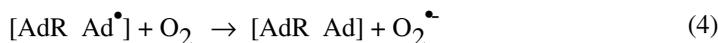


In this reaction, adrenodoxin (Ad) is the substrate of the reductase. Consistent with this, the dependence of the rate of NADPH oxidation on [adrenodoxin] shows Michaelis-Menten kinetics (Hanukoglu et al., 1993).

In the third step, reduced adrenodoxin donates its electron to O<sub>2</sub>, generating superoxide. Kinetic analyses suggest that this reaction takes place while adrenodoxin remains bound to AdR (Hanukoglu et al., 1993):



This reaction is probably immediately followed by the transfer of the second electron of AdR to adrenodoxin and reduction of a second molecule of O<sub>2</sub>:



After this step, the next cycle of events starts over again with reaction (1).

The interactions of AdR and adrenodoxin are dependent on their redox status. The mid-point potential of adrenodoxin reductase is -274 mV (Table 3) and is not significantly affected by the binding of adrenodoxin (Lambeth and Kamin, 1976). However, the redox potential of adrenodoxin shifts by at least 40 mV (from about -290 mV to -330 mV) upon its binding to reductase (Table 3) (Grinberg et al., 2000; Lambeth and Kamin, 1979). The redox potential for the oxygen/superoxide couple is -330 mV (Table 3). Thus, AdR-adrenodoxin<sub>red</sub> complex would have a more favorable potential to reduce O<sub>2</sub> than unbound adrenodoxin<sub>red</sub>. It has been noted that adrenodoxin functions as an automatic "electron gun," constantly being loaded and fired, until electron supply via NADPH is exhausted (Hanukoglu et al., 1993).

After NADPH depletion, reduced adrenodoxin molecules oxidize at a slower rate that has been referred to as "auto-oxidation" (Hanukoglu et al., 1993). Auto-oxidation

**Table 3** Standard reduction potentials of the redox couples reviewed.

Oxidized form	Reduced form	Ligand	E <sup>o'</sup> (mV)	Reference
NADP <sup>+</sup>	NADPH	–	–320	–
FAD	FADH <sub>2</sub>	–	–219	–
AdR	AdR <sub>red</sub>	–	–274	[Chu, 1973 #637]
AdR	AdR <sub>red</sub>	Adrenodoxin	–292 ± 8 (pH 7.5)	(Lambeth et al., 1976)
Adrenodoxin	Adrenodoxin <sub>red</sub>	–	–273	(Huang and Kimura, 1973)
			–291 ± 18 (pH 7.5)	(Lambeth et al., 1976)
Adrenodoxin	Adrenodoxin <sub>red</sub>	AdR <sub>red</sub>	–331 ± 5 (pH 7.5)	(Lambeth et al., 1976)
				(Lambeth and Kamin, 1979)
P450scc	P450scc <sub>red</sub>	–	–412 ± 2 (pH 7.4)	(Light and Orme-Johnson, 1981)
P450scc	P450scc <sub>red</sub>	Cholesterol	–284	(Lambeth and Pember, 1983)
			–305 ± 1 (pH 7.4)	(Light and Orme-Johnson, 1981)
P450scc	P450scc <sub>red</sub>	Cholesterol + adrenodoxin	–314	(Lambeth and Pember, 1983)
P450scc	P450scc <sub>red</sub>	Pregnenolone	–333 ± 3 (pH 7.4)	(Light and Orme-Johnson, 1981)
O <sub>2</sub>	O <sub>2</sub> <sup>•–</sup> (superoxide)	–	–160	(Schafer and Buettner, 2001)
			–330 (at 1 atm.)	
Ascorbate <sup>•–</sup> , H <sup>+</sup>	Ascorbate	–	+282	(Buettner, 1993)
Tocopheryl <sup>•</sup> , H <sup>+</sup>	Tocopherol	–	+500	(Buettner, 1993)
Superoxide	H <sub>2</sub> O <sub>2</sub>	–	+940	(Buettner, 1993)

AdR: Adrenodoxin reductase, AdR<sub>red</sub>: Reduced adrenodoxin reductase.

Unless indicated, all measurements are at pH = 7.0. The proteins are all from bovine sources.

Reduction potential is a measure of the tendency of each couple to accept or donate an electron. In general, a reduced form with a lower potential is capable of donating an electron to the oxidized form of a couple with a higher potential. For example, NADPH (–320 mV) can reduce FAD (–219 mV), but not vice versa.

kinetics fit first-order reaction that is highly dependent on temperature (Hanukoglu et al., 1993). Adrenodoxin<sub>red</sub> has a lower affinity for AdR than oxidized adrenodoxin (Lambeth and Kamin, 1979). Thus, in absence of electron supply from AdR, adrenodoxin<sub>red</sub> would tend to dissociate from AdR. Because unbound adrenodoxin<sub>red</sub> has a higher (less negative) redox potential than AdR–adrenodoxin<sub>red</sub> complex, it should be less effective at reducing O<sub>2</sub> (compare values in Table 3). Consequently, the rate of superoxide production drops drastically during auto-oxidation (Hanukoglu et al., 1993).

Oxidation of NADPH by AdR–adrenodoxin couple is associated with inactivation of adrenodoxin (Rapoport et al., 1995). This inactivation was inhibited by the addition of superoxide dismutase (SOD) but not of catalase, indicating that the agent responsible for the inactivation was superoxide radical and not H<sub>2</sub>O<sub>2</sub> (Rapoport et al., 1995).

As part of the mechanism of stimulation of steroidogenesis, trophic hormones may cause a transient increase in mitochondrial matrix calcium ions (Ca<sup>2+</sup>) (Spat and Hunyady, 2004). Submillimolar concentrations of Ca<sup>2+</sup> stimulate electron leakage from the reductase–adrenodoxin couple, while MgCl<sub>2</sub> seem to have no effect at similar concentrations (Hanukoglu et al., 1993). In the presence of 100 mM NaCl, micromolar concentrations of CaCl<sub>2</sub> have been reported to inhibit P450scc activity in Tween 20 or in phospholipid vesicles (Hanukoglu et al., 1981a). This inhibition may be partly due to the electron leakage stimulating effect of Ca<sup>2+</sup>. The interactions of adrenodoxin with mitochondrial P450s is greatly affected by the ionic strength of the solution (Hanukoglu et al., 1981a, 1981b; Lambeth and Kriengsiri, 1985; Schiffler et al., 2004). Yet, the effect of Ca<sup>2+</sup> at low concentrations indicates an ion-specific effect independent of ionic strength.

**Electron leakage from the P450s.** In reconstituted mitochondrial P450 systems, electron leakage reactions have been studied with both P450scc, specific for cholesterol side-chain-cleavage, and P450c11, specific for steroid 11 $\beta$ -hydroxylation (Hanukoglu et al., 1993; Rapoport et al., 1995). Addition of either of these P450s increases superoxide production significantly above that observed by reductase and adrenodoxin couple (Hanukoglu et al., 1993; Rapoport et al., 1995).

In the absence of its substrate cholesterol, P450scc has been observed to be more active than adrenodoxin in reducing O<sub>2</sub> to superoxide, with a maximal effect at a much lower concentration (0.3  $\mu$ M vs. >7  $\mu$ M for adrenodoxin) (Hanukoglu et al., 1993). In adrenal mitochondria the concentrations of P450scc and adrenodoxin are similar (Hanukoglu and Hanukoglu, 1986). In a system with this molar ratio of the two proteins, electrons appeared to leak mainly through P450scc and not through adrenodoxin (Hanukoglu et al., 1993).

Ideally, in the absence of its substrate, P450 should have a lower reduction potential to minimize its futile reduction and to avoid ROS production. Indeed, under anaerobic conditions (without O<sub>2</sub>), the reduction potential of substrate free P450scc (-412 mV) is lower than that of P450scc-cholesterol complex (-305 mV) (Table 3). However, substrate-free P450scc is reduced easily by adrenodoxin under aerobic conditions and leads to production of superoxide (Hanukoglu et al., 1993). A possible explanation for this apparent discrepancy is that the binding of O<sub>2</sub> may increase the redox potential of P450scc. Adrenodoxin in oxidized or reduced forms does not have a major effect on the reduction potential of P450scc (Table 3) (Lambeth and Pember, 1983). Many microsomal P450s have been observed to function as NADPH oxidase reducing oxygen in the absence of a substrate, and producing superoxide anion, hydrogen peroxide, or water (Ding et al., 1991; Kohno et al., 2005; Puntarulo and Cederbaum, 1996). NADPH oxidase activity of some of these microsomal P450s (e.g., Ding et al., 1991), is much higher than that of P450scc.

**Effects of steroids on electron leakage.** A study that examined ROS formation in reconstituted mitochondrial P450 systems revealed that both P450scc and P450c11 generate ROS during their catalytic function of steroid hydroxylation (Rapoport et al., 1995). The assay system in this study included SOD and catalase to convert superoxide generated during reactions to H<sub>2</sub>O<sub>2</sub>. The steroid product and H<sub>2</sub>O<sub>2</sub> were then assayed after terminating the reactions (Rapoport et al., 1994, 1995). While in the P450scc system at most 15% of the total electron flow was directed to ROS formation; in the P450c11 system 32–43% of the electrons were consumed in ROS generation (Rapoport et al., 1995). In these studies, P450scc reactions were carried out at 37°C, and P450c11 reactions at 30°C because of the temperature sensitivity of P450c11. Thus, the difference between the leakage rates of these P450s would be further magnified at 37°C reactions for P450c11.

Examination of the effects of substrate and other steroids revealed further differences between the two mitochondrial P450s. P450scc system produced less H<sub>2</sub>O<sub>2</sub> during cholesterol substrate hydroxylation than that observed in the absence of substrate. In other words, the coupling of the P450scc system was greater during cholesterol metabolism (Rapoport et al., 1995). Earlier stopped-flow measurements also indicated that cholesterol inhibits P450scc autooxidation (Tuckey and Kamin, 1983). In contrast, P450c11 system produced more H<sub>2</sub>O<sub>2</sub> during substrate deoxycorticosterone metabolism (Rapoport et al., 1995). The product of P450scc, pregnenolone, had no stimulatory effect on futile NADPH oxidation by the P450scc system. In contrast, 11 $\beta$ -hydroxylated products of P450c11, and other steroids with a strict stereo-specificity, strongly stimulated electron leakage from P450c11, but not from P450scc (Rapoport et al., 1995). These results overall indicated

that P450c11 catalyzed steroid hydroxylation is poorly coupled relative to the P450scc activity.

Analyses of the steroid effects on P450c11 reactions (Rapoport et al., 1995) show that steroids can be classified into four groups based on their effects and their structures (Table 4):

1. Leakage stimulatory substrates: C-21 steroids with a 3-keto group and 4-ene double bond. Examples: Deoxycortisol, deoxycorticosterone.
2. Leakage stimulatory products or analogs: C-19 and C-21 steroids with an 11 $\beta$ -hydroxy and a 3-keto group and 4-ene double bond that do not appear to be a substrate for P450c11. Examples: 11 $\beta$ -OH-testosterone, corticosterone, progesterone, and cortisol.
3. Leakage inhibitory steroids: C-19 and C-21 steroids with an 11 $\alpha$ -hydroxy or an 11-keto group, a 3-keto group and 4-ene double bond. Examples: 11 $\alpha$ -OH-testosterone and 11 $\alpha$ -OH-cortisol.
4. Steroids without an effect: C-19 steroids without a 3-keto group or 4-ene double bond. Examples: 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol, 5 $\alpha$ -androstanedione, and 4-androstene-3 $\beta$ ,17 $\beta$ -diol.

In other P450 systems, various substrates and substrate analogs show wide variation in their effects on the coupling of the electron transfer process (Jung et al., 2002; Shertzer et al., 2004a, 2004b).

Substrates and analogs may influence the coupling of the electron transfer by several mechanisms; e.g., changing accessibility of the heme pocket to water and modifying the interaction of O<sub>2</sub> with the heme iron of P450. To explain the inhibition of electron leakage from P450scc during substrate cholesterol metabolism, in analogy with the P450cam structure (Poulos et al., 1985), it has been suggested that the substrate may fully occupy the active site, excluding water and decreasing the polarity of the microenvironment of the FeO<sub>2</sub> complex, and consequently inhibit the release of superoxide (Hanukoglu et al., 1993). Studies of P450cam heme pocket using high-pressure stopped-flow technique indicate that a suboptimal fit of the substrate in the heme pocket increases the mobility of the substrate, facilitates the access for water molecules, disturbs the tight structural coupling for a specific proton transfer and, thus, may lead to the formation of hydrogen peroxide or of water in the oxidase reaction rather than substrate hydroxylation (Jung et al., 2002). The substrate cholesterol apparently stabilizes P450scc-O<sub>2</sub> complex, saving it for reduction by

**Table 4** Classification of steroids based on their effect on P450c11 activity and electron leakage.

Effect on leakage	Steroid type	Functional groups	Examples
Stimulatory substrate	C-21	3-keto, 4-ene 11-deoxy	Deoxycortisol
Stimulatory analog	C-19 or C-21	3-keto, 4-ene 11 $\beta$ -hydroxy	Deoxycorticosterone Cortisol
Inhibitory	C-19 or C-21	3-keto, 4-ene 11 $\alpha$ -hydroxy	Corticosterone Progesterone 11 $\beta$ -OH-Testosterone 11 $\alpha$ -OH-Cortisol
No effect	C-19	3 $\beta$ -OH or 5 $\alpha$	11 $\alpha$ -OH-Testosterone 5 $\alpha$ -Androstane-3 $\beta$ ,17 $\beta$ -diol 5 $\alpha$ -Androstanedione 4-Androstene-3 $\beta$ ,17 $\beta$ -diol

The table is based on results presented by Rapoport et al. (1995).

the second electron (Hanukoglu et al., 1993). In contrast, the substrates of P450c11 stimulate the autooxidation of P450c11-O<sub>2</sub> complex, releasing superoxide. The reduction of leakage by 11 $\alpha$ -OH steroids may result from inhibition of P450c11-O<sub>2</sub> complex formation or inhibition of the autooxidation of this complex. The steroids that have no effect on electron leakage may not enter into the substrate pocket of P450, or simply not affect the heme-O<sub>2</sub> interaction in the active site.

### **Mitochondrial P450 Systems as a Source of ROS in Cultured Cells**

The studies reviewed previously showed that mitochondrial P450 systems reconstituted with purified proteins can generate reactive oxygen species. The question is then whether these results represent physiologically significant phenomenon in the natural environment of the mitochondria of steroidogenic cells. Studies in cultured cells, reviewed in the following paragraphs, reveal major similarities between the behavior of the mitochondrial P450 system proteins in reconstituted systems and within cells in culture.

**Effects of overexpression on ROS dependent apoptosis.** To examine the effect of over-expression of adrenodoxin and P450<sub>scc</sub>, Derouet-Humbert et al. (2005) transiently cotransfected an expression plasmid encoding for human adrenodoxin, bovine adrenodoxin, or human CYP11A1 into 11 tumor-derived and non-tumorigenic cell lines. The results of their studies showed that overexpression of either human or bovine adrenodoxin led to a decrease in cell viability in all cell lines tested. Adrenodoxin expression enhanced oxidation of dihydroethidium to ethidium, providing evidence for the generation of ROS in the cell lines. In order to function as an “electron gun,” adrenodoxin is dependent on adrenodoxin reductase (AdR) to receive reducing equivalents from NADPH, which are then donated to O<sub>2</sub> (Hanukoglu et al., 1993). AdR is ubiquitously expressed in different cells. Thus, apparently endogenously expressed AdR was sufficient to allow overly-expressed adrenodoxin to function as a source of ROS in transfected cells.

Formation of ROS within mitochondria is one of the major internal triggers for apoptotic cell death (Bras et al., 2005). An early step in this process is the release of cytochrome c from within the inter-membrane space of the mitochondria. Cytochrome c aggregates with apoptosis-protease-activating factor 1 (Apaf-1), and procaspase-9 forming apoptosomes. Caspase-9 is cleaved and activated by the apoptosomes and then it activates downstream effector caspases (caspase-3 and -7), spreading a cascade of proteolytic activity that leads to digestion of structural proteins in the cytoplasm and eventual phagocytosis of the cell (Bras et al., 2005).

Consistent with the process of apoptosis, overexpression of adrenodoxin was shown to disrupt mitochondrial transmembrane potential, indicating breakdown of the outer mitochondrial membrane and increased caspase activity in the cell (Derouet-Humbert et al., 2005). Overexpression of an apo-mutant of adrenodoxin (C46S) that does not have the iron-sulfur (2Fe-2S) cluster essential for electron transfer did not cause apoptosis (Derouet-Humbert et al., 2005). This control experiment further strengthens the conclusion that the cause of apoptosis is the superoxide production by adrenodoxin.

It has been suggested that adrenodoxin reductase (AdR) contributes to p53-mediated apoptosis through the generation of oxidative stress in mitochondria (Hwang et al., 2001). This suggestion was based on the observation that AdR gene is highly induced by the chemotherapeutic agent 5-fluorouracil (5-FU) mediated by the p53 protein in HCT116 cell lines (Hwang et al., 2001; Liu and Chen, 2002). However, induction of AdR expression failed to show any effect on cell proliferation (Liu and Chen, 2002), and stable or

transient overexpression of AdR did not decrease cell viability in different cell lines (Derouet-Humbert et al., 2005). As noted previously, AdR by itself does not significantly oxidize NADPH. Only in the presence of adrenodoxin AdR serves as a conduit for electrons coming from NADPH (Hanukoglu et al., 1993). Consistent with these findings, AdR over-expression by itself is not associated with increased ROS formation or ROS triggered apoptosis (Derouet-Humbert et al., 2005).

Overexpression of human CYP11A1 that encodes P450<sub>scc</sub> showed variable effects in 11 cell lines, leading the authors to conclude that “difference in the availability of mitochondrial cholesterol might contribute to the observed tissue specificity of CYP11A1-induced apoptosis” (Derouet-Humbert et al., 2005). Again, these observations are consistent with the results observed in reconstituted systems.

**Effects of steroids and ROS on mitochondrial P450s.** Studies using purified enzymes showed major differences in the capacity of different mitochondrial P450s to generate and be affected by ROS (see previous sections). Studies in cultured cells show similar differences.

In cultured adrenocortical cells the activity of P450<sub>c11</sub> decreases rapidly as a result of oxidative damage (Hornsby and Crivello, 1983; Hornsby, 1980, 1989). The loss of the activity of P450<sub>c11</sub> is not accompanied by loss of steroid synthesis, indicating that P450<sub>scc</sub> continues to function with little or no damage under the same conditions. These findings are consistent with the observation that P450<sub>c11</sub> is much more leaky than P450<sub>scc</sub> in reconstituted systems (Rapoport et al., 1995). Thus, the inactivation of P450<sub>c11</sub> in cultured cells may directly result from its much greater propensity to generate harmful oxygen radicals. Another possibility is that inherently, P450<sub>c11</sub> may be more prone than P450<sub>scc</sub> to denaturation. P450<sub>c11</sub> is more susceptible to degradation during NADPH-dependent lipid peroxidation in bovine adrenal cortex mitochondria (Klimek et al., 1983).

The half-life of P450<sub>c11</sub> in cultured adrenocortical cells is drastically shortened by substrates of P450<sub>c11</sub> or 11 $\beta$ -hydroxylated steroids, including products of P450<sub>c11</sub> (Hornsby, 1980). In contrast, 11 $\alpha$ -hydroxylated steroids or 11-ketosteroids did not affect 11 $\beta$ -hydroxylase activity (Hornsby, 1980). This structural specificity of the steroids in inactivation of P450<sub>c11</sub> in cultured cells is identical to that observed in stimulation of electron leakage from P450<sub>c11</sub> in reconstituted systems (Table 4). The stereospecificity of the effects in both systems (e.g., harmless 11 $\alpha$ -OH-testosterone vs. harmful 11 $\beta$ -OH-testosterone) establish unequivocally that the results in cultured cells are specifically a result of the interaction of the steroids with the enzyme P450<sub>c11</sub> itself, stimulating production of ROS by P450<sub>c11</sub> during the process of electron transfer.

In steroidogenic cells there are no major vesicular stores of steroid products, as steroids are rapidly secreted into the blood stream after synthesis. Thus, the effects of the products in stimulating ROS production by P450<sub>c11</sub> should not be a major problem in normal physiology. However, experimental observations noted previously raise a caveat that steroidal drugs may have similar effects stimulating ROS production by steroid metabolizing enzymes.

An example of a ROS-associated pathological action of a steroid is the effect of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>), the active form of vitamin D<sub>3</sub>, in breast cancer cells. The addition of this steroid derivative induces apoptosis in human breast cancer MCF-7 cells by disruption of mitochondrial function, associated with cytochrome c release, and production of reactive oxygen species (Narvaez and Welsh, 2001; Narvaez et al., 2003). The exact mechanism of the effect of this steroid has not yet been elucidated,

but one possibility is that it may stimulate ROS production similar to the effects observed with other steroids.

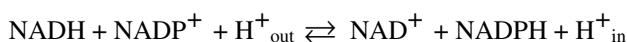
### Protective Mechanisms to Minimize ROS Production and Damage

The studies reviewed previously establish that mitochondrial P450 systems produce ROS. According to the *in vitro* studies, if NADPH is constantly available to the mitochondrial P450 systems then these oxidize NADPH in a futile cycle, generating harmful oxyradicals without coupling to steroid metabolism. The next section on the regulation of NADPH in the mitochondria of steroidogenic cells concludes that NADPH supply is not tightly linked to steroid biosynthesis. Thus, production of ROS by mitochondrial P450 systems in steroidogenic cells appears to be an inevitable phenomenon. The following section reviews antioxidants that may be involved in scavenging ROS in steroidogenic cells.

**Regulation of NADPH availability for mitochondrial P450 systems.** The first step of steroid hormone biosynthesis in all steroidogenic tissues is the cleavage of the side-chain of cholesterol by P450<sub>scc</sub> that produces pregnenolone (Hanukoglu, 1992; Miller, 2005; Payne and Hales, 2004). This initial reaction is dependent on the supply of cholesterol from the cytoplasm. The transfer of cholesterol from the cytoplasmic vesicles to the mitochondria is tightly regulated by trophic hormone stimulated mechanisms that involve steroidogenic acute regulatory protein (StAR) in most steroidogenic cells (Jefcoate, 2002; Niswender, 2002; Strauss et al., 2003). In the placenta, a different START protein (MLN64) is involved in the transport of cholesterol to the mitochondria (Tuckey, 2005).

In steroidogenic cells, the supply of cholesterol to mitochondria can be tightly coupled with trophic hormonal signals, because the supplied cholesterol is uniquely used in steroid biosynthesis. In contrast to cholesterol, the delivery of NADPH cannot be uniquely coupled to steroid hormone biosynthesis because, in addition to serving as an electron donor for P450 systems, NADPH is also used by a multitude of reactions within the mitochondria (Berger et al., 2004; Di Lisa and Ziegler, 2001; Hanukoglu and Rapoport, 1995; Hoek and Rydstrom, 1988; Kirsch and De Groot, 2001). Thus, continuous maintenance of a basal level of NADPH in the mitochondrial matrix is essential for normal function.

NADPH that supplies electrons to the mitochondrial P450 systems may be generated by several alternative routes (Hanukoglu and Rapoport, 1995). In the short-term, the synthesis of NADPH can be regulated by modulating the activities of the enzymes in these routes. The intra-mitochondrial levels of NADPH are co-regulated with NADH generated by the Krebs cycle, fatty acid  $\beta$ -oxidation and other redox reactions inside the mitochondria. The enzyme nicotinamide nucleotide transhydrogenase catalyzes the interconversion of NADH and NADPH according to the reaction (Hatefi and Yamaguchi, 1996; Jackson, 2003):



This enzyme also functions as a proton pump in the inner mitochondrial membrane. In the forward direction, NADPH synthesis is coupled with  $\text{H}^+$  translocation from the cytoplasmic side into the matrix of the mitochondria, and the converse occurs for the reverse reaction. Thus, because of its coupling to the proton electrochemical gradient across the inner mitochondrial membrane, the transhydrogenase activity (consequently NADPH production) is linked to the functioning of oxidative phosphorylation chain.

Currently, there is no precise information on the absolute concentrations of the pyridine nucleotides NADH and NADPH in the mitochondrial matrix where the steroidogenic

enzymes are located. Some early studies suggested that tropic hormones regulate the Krebs cycle and NADPH production (Peron et al., 1975). Studies using single cell fluorimetric technique revealed that in rat adrenal glomerulosa cells, angiotensin II and cytoplasmic signal  $Ca^{2+}$  evoke an increase in the level of reduced mitochondrial pyridine nucleotides (Spat and Hunyady, 2004; Spat and Pitter, 2004). These studies established that the supply of NADPH can be enhanced to meet the demands of trophic hormone stimulated steroidogenesis.

Combining the previous observations, the general strategy of NADPH regulation in mitochondria appears to be maintenance of low basal levels that are augmented in bursts in response to steroidogenic trigger signals initiated by trophic hormones. While cholesterol supply is regulated by “on-off” switch type mechanisms, NADPH synthesis is regulated by mechanisms that function as a graded rheostat.

Studies in reconstituted systems for both P450<sub>scc</sub> and P450<sub>c11</sub> demonstrated that the magnitude of electron leak from these systems depends on the concentration of NADPH even in the micromolar range (Rapoport et al., 1995). Thus, even in the presence of low levels of NADPH the systems would be expected to leak electrons.

A study that examined the redox state of adrenodoxin by ESR spectroscopy concluded that adrenodoxin remains fully reduced in hypophysectomized rat adrenals with or without ACTH treatment (Williams-Smith et al., 1976). This interpretation requires a caveat that cellular metabolism could have changed within minutes after induction of anesthesia and prior to the removal of the adrenal. If these findings reflect normal physiological status, then adrenodoxin may be a source of ROS in absence of steroid metabolism.

An important conclusion that can be derived from the adrenodoxin overexpression studies reviewed previously is that, apparently NADPH in the mitochondrial matrix is directly being oxidized by the AdR-adrenodoxin couple even in the absence of a P450 that could receive the electrons channeled via adrenodoxin. In other words, NADPH supply does not appear to be a rate-limiting factor for the AdR-adrenodoxin couple to function as an artificial NADPH oxidase generated by the adrenodoxin overexpression. This conclusion may be questioned, noting that the cells used in overexpression studies are non-steroidogenic cells. Yet, the mitochondrial P450 system electron transfer proteins are ubiquitously expressed in different tissues. Therefore, the consistent effect of adrenodoxin overexpression in 11 different cell types probably reflects a general status.

In addition to short-term mechanisms of enzyme activation and intermediate substrate metabolism, NADPH biosynthesis may also be regulated by long-term mechanisms at the level of the expression of the enzymes in the pathways of NAD(P)H production. ACTH was shown to induce the expression of mitochondrial mRNAs encoding subunits of oxidative phosphorylation system enzymes, in parallel with the induction of steroidogenic enzyme genes in adrenocortical cells in culture (Raikhinsein and Hanukoglu, 1993, 1994). In bovine corpora lutea, a low but significant correlation was observed between mitochondrial P450 system enzymes and a cytochrome oxidase subunit (Hanukoglu and Hanukoglu, 1986). In mouse ovary, the activities of isocitrate dehydrogenase and other enzymes of glucose metabolism vary in parallel with the steroidogenic capacity of the ovary (Chapman et al., 1992).

**Antioxidants in steroidogenic tissues.** The major source of free radicals, such as superoxide and other types of ROS, in the cell, is the electron transfer reactions of the mitochondrial oxidative phosphorylation chain (Andreyev et al., 2005; Turrens, 2003). The ROS generated by the respiratory chain can damage proteins, lipids, and DNA. There are several lines of evidence that these changes, but especially damage to the mitochondrial

DNA (mtDNA), are responsible for the process of aging (Barja, 2004; Melov, 2004; Vina et al., 2005). The rate of mitochondrial ROS generation is related to the level of oxidative damage to mtDNA and is inversely correlated with maximum longevity in higher vertebrates (Barja, 2004). To prevent the harmful effects of ROS, mitochondria have a rich repertoire of enzymes (e.g., manganese superoxide dismutase (Mn-SOD), catalase, glutathione peroxidase, and glutathione reductase) and small antioxidant molecules (e.g., ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene) that can neutralize or scavenge superoxide and other ROS (Andreyev et al., 2005).

The studies reviewed previously have indicated that in steroidogenic tissues, the mitochondrial P450 systems can represent an additional burden over the common oxidative stress of the respiratory chain. Thus, steroidogenic tissues would be expected to have a strong dependence on antioxidants as a defense mechanism. Indeed, the studies reviewed next indicate that the normal function of steroidogenic tissues is dependent on antioxidants. In addition to their function as antioxidants, molecules such as ascorbic acid and  $\beta$ -carotene fulfill biological roles independent of interaction with oxygen radicals. For example, retinoid derivatives of  $\beta$ -carotene regulate gene transcription in cells by binding to nuclear retinoid receptor (Lane and Bailey, 2005). Therefore, the effects of antioxidant molecules should be examined to ascertain that these are due to antioxidant properties of the molecule and not secondary effects of other biological actions.

**Antioxidants in the adrenal cortex.** In bovine adrenal cortex cells in culture, P450c11, but not P450scc, undergoes rapid degradation in the absence of antioxidants and this degradation can be prevented by the addition of ascorbic acid and  $\alpha$ -tocopherol (Hanukoglu et al., 1990a; Hornsby et al., 1985; Hornsby, 1989). The requirement for antioxidants for P450c11 stability (but not for P450scc) in cultured cells is consistent with the observations in reconstituted systems that P450c11 leaks electrons much more than P450scc (Rapoport et al., 1995). Chronic *in vivo* administration of ACTH in rats was observed to reduce plasma aldosterone, down-regulate CYP11B2 mRNA, and reduce P450aldo activity (Lehoux et al., 1998; Suwa et al., 2000). The induced decrease in CYP11B2 mRNA level was recovered with co-administration of vitamin E or DMSO possibly through their antioxidant actions (Suwa et al., 2000).

In rat adrenals, measurement of the total SOD activity and the activity of the mitochondrial manganese superoxide dismutase (Mn-SOD) indicated that Mn-SOD encoded by the SOD2 gene accounts for all the SOD activity (Raza et al., 2005). Mn-SOD is induced by ACTH in bovine adrenocortical cells (Chinn et al., 2002). Chronic ACTH administration in rats increased Mn-SOD expression, but decreased glutathione peroxidase mRNA in the adrenal capsules (Raza et al., 2005; Suwa et al., 2000). ACTH administration, together with antioxidant vitamin E or DMSO, inhibited ACTH induction of Mn-SOD (Suwa et al., 2000). One possible explanation for this finding is that antioxidants reduce the oxidative stress, consequently reducing the stimulus for mitochondrial ROS mediated induction of Mn-SOD. Recent findings indicate that mitochondrial ROS activate a mitochondrion-to-nucleus signal relay pathway in which the serine/threonine protein kinase D (PKD) activates the NF-kappaB transcription factor, leading to induction of SOD2 expression (Storz et al., 2005).

In addition to natural antioxidants, a protective effect against the oxidative stress of steroid biosynthesis has been demonstrated using artificial antioxidants. The addition of an antioxidant drug, silibinin, at a low dose, potentiated ACTH-stimulated secretion of corticosteroids in hyperplastic adrenocortical cells, leading the authors to conclude that the effect of silibinin is presumably due to the antioxidant property of the drug (Racz

et al., 1990). In bovine kidney proximal tubule cells in primary culture, mitochondrial vitamin D hydroxylase activities were found to be inversely proportional to the increase in mitochondrial membrane lipid hyperperoxide production (Crivello, 1988). Pretreatment of the culture with antioxidants, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), drastically reduced lipid hyperperoxide production (Crivello, 1988).

Whereas vitamin A related  $\beta$ -carotene, and vitamin E related  $\alpha$ -tocopherol are lipid-soluble, vitamin C (ascorbic acid) is a water-soluble antioxidant. Among all organs in the body, the concentration of ascorbic acid is highest in the steroidogenic tissues, adrenal cortex, and corpus luteum (Hornig, 1975; Mahan et al., 2004; Rapoport et al., 1998). ACTH stimulated cortisol secretion from the guinea pig adrenal is accompanied by a decrease in the levels of ascorbate in the adrenal, but not in other tissues or plasma (Laney et al., 1990). In rats, vitamin A depletion causes a secondary deficiency of ascorbic acid in the adrenal, leading to adrenocortical degeneration, and ascorbate supplement prevents degeneration (Gruber et al., 1976). Ascorbate depletion prevents aldosterone stimulation by sodium deficiency in the guinea pig (Redmann et al., 1995).

An important action of ascorbate as an antioxidant is the recycling of  $\alpha$ -tocopheryl radical (Buettner, 1993; May, 1999; Rose and Bode, 1993). Thus, in the guinea pig adrenal cortex, actions of ascorbate were shown to be related to the mitochondrial tocopherol content in the different zones of the cortex (Staats and Colby, 1989).

**Antioxidants in the ovary.** In the ovary, the types and quantities of steroids synthesized differ by the stage of the estrus cycle of animals. After ovulation, the follicle enters the luteal phase, during which granulosa and theca cells of the follicle proliferate to form corpus luteum, and synthesize and secrete progesterone at high levels (nmol/L range) to prepare the uterus for implantation. If there is no pregnancy, then corpus luteum regresses by an apoptotic process (McCracken et al., 1999; Niswender, 2002; Tilly, 1996; Zheng et al., 1994). During the luteal phase, the increase in progesterone secretion reflects proliferation of steroidogenic cells and a major increase in the expression of the steroidogenic enzymes, primarily mitochondrial cholesterol side-chain cleavage P450<sub>scc</sub>. The final stage of corpus luteum regression is characterized by a decrease in the levels of the P450<sub>scc</sub> system proteins (Devoto et al., 2002; Hanukoglu, 1992; Rapoport et al., 1998; Rodgers et al., 1987).

Reactive oxygen species (ROS) and antioxidants appear to have two contrasting effects in the development and regression of the corpus luteum. During the initial phase of development and cell proliferation with enhanced progesterone synthesis, ROS generated by the mitochondrial P450<sub>scc</sub> system represent a harmful byproduct of steroidogenesis. Therefore, as a defense against these ROS, antioxidants would be expected to rise in parallel with the steroidogenic capacity of the cells. In contrast, at the stage of luteolysis, free radicals could play a functional role in apoptotic cell death and contribute to regression of corpus luteum (Behrman et al., 2001; Foyouzi et al., 2005; Niswender et al., 2000). Thus, at the final stage of corpus luteum, antioxidant levels would be expected to decrease, allowing ROS to contribute to apoptosis and regression of the corpus luteum. The findings reviewed in the following paragraphs for the antioxidant enzymes SOD, catalase, and glutathione peroxidase are, in general, consistent with these expected trends. The findings for the small antioxidant molecules show that the levels of these antioxidants change significantly during corpus luteum development, yet there does not appear to be a consistent pattern indicating complex functional and regulatory roles for these. It should be emphasized the ROS is only one group of a large number of factors that contribute to luteal regression (Amsterdam et al., 2003; Hussein, 2005; McCracken et al., 1999).

Determination of the activities of the antioxidant enzymes, SOD and catalase, showed that these are correlated with the plasma progesterone levels during the bovine estrus cycle (Rapoport et al., 1998). In bovine corpora lutea, the activities of SOD and catalase rise and reach a peak at about day 16 of the cycle and then drop in parallel with the levels of P450<sub>scc</sub> and adrenodoxin (Rapoport et al., 1998). In sheep corpora lutea, cytosolic Cu-Zn-SOD, mitochondrial Mn-SOD, and glutathione peroxidase activities rise while nitric oxide synthase (NOS) decreases during the luteal phase (Al-Gubory et al., 2005). During the human menstrual cycle, the activity of Mn-SOD, as well as the levels of its mRNA, were shown to increase during late luteal phase, indicating that increased enzyme activity is a result of enhanced expression of the SOD2 gene (Sugino et al., 2000). However, while Cu-Zn-SOD activity and mRNA dropped during luteal regression, Mn-SOD activity and mRNA showed further increases, suggesting that these two enzymes play different roles in regulating luteal function (Sugino et al., 2000). In cultured ovarian cancer cells, ROS stress induced Mn-SOD expression; suppression of Mn-SOD expression by siRNA caused an increase in superoxide and promoted cell proliferation *in vitro* and tumor growth *in vivo* (Hu et al., 2005). These findings emphasize the role of Mn-SOD in coping with ROS generated in the mitochondria.

In addition to SOD, catalase, and glutathione peroxidases, a family of more recently identified peroxidases named peroxiredoxins may also be involved in protection against ROS damage in the mitochondria. In Chinese hamster ovary cells, overexpression of mitochondrial peroxiredoxin 5 showed significant protective effect on mtDNA against lesions caused by exogenously added hydrogen peroxide (Banmeyer et al., 2005).

There are several independent lines of evidence that trophic hormones that are involved in the stimulation of corpus luteum steroidogenesis are also involved in inducing antioxidant enzymes. Lutropin (human luteinizing hormone, LH) that stimulates follicle development, ovulation, and corpus luteum formation was shown to induce SOD in rat ovary (Laloraya et al., 1988). Prolactin that functions as a luteotropic hormone in the rat also stimulates Cu-Zn-SOD and Mn-SOD mRNA expression in rat luteal cells (Sugino et al., 1998).

The mechanism of gonadotropin induction of antioxidant enzymes is not known yet. Hormones such as LH may activate the expression of these enzymes via a direct second-messenger mediated pathway. Alternatively, induction of antioxidant enzymes may be a secondary effect of enhanced ROS production that accompanies enhanced steroidogenic activity. As noted previously in the section on the adrenal cortex, mitochondrial ROS can activate a mitochondrion-to-nucleus signal to influence gene expression.

Treatment of rat corpus luteum cells with LH that stimulates progesterone secretion has been observed also to increase superoxide generation in a dose-dependent manner (Sawada and Carlson, 1996). These findings have been interpreted to suggest that superoxide may be involved in the mechanism of luteotropic response to LH (Sawada and Carlson, 1996). However, superoxide production is probably stimulated by LH, as a side-reaction, a byproduct, of the LH stimulation of steroidogenesis. As noted previously, trophic hormone stimulation in adrenal cells is associated with increased NADPH synthesis. Similarly, LH probably enhances NADPH levels in luteal cells. As a consequence of LH-stimulated steroidogenesis, superoxide production would be expected to increase as well. Studies in reconstituted systems using purified proteins do not provide evidence for the involvement of exogenous ROS in the biosynthetic reactions catalyzed by the mitochondrial P450s. On the contrary, exogenous ROS are detrimental to the function of the P450

system proteins. Similarly, in cultured luteal cells, high levels of superoxide are associated with inhibition of progesterone secretion (Sawada and Carlson, 1996).

Among the small molecule antioxidants, ascorbate is present at highest concentrations in corpora lutea, similar to the high levels observed in the adrenal cortex (Table 5). In bovine corpus luteum, the levels of ascorbate increase during the luteal phase and then drop during regression (Rapoport et al., 1998). In rat luteal cells, stimulation with luteinizing hormone (LH) or luteolytic factor, prostaglandin (PG)  $F_{2\alpha}$  rapidly causes depletion of ascorbic acid (Musicki et al., 1996). In the corpus luteum of the pseudopregnant rat, acute treatment with  $PGF_{2\alpha}$  decreased luteal vitamin C levels coincident with transient lipid peroxidation and a fall in serum progesterone levels (Aten et al., 1992). Vitamin C is taken up into the cell by specific transporter proteins SVCT1 and SVCT2 (Wilson, 2005). In rat granulosa cells in culture, gonadotropic hormone FSH and insulin-like growth factor I (IGF-I) stimulate uptake of ascorbic acid (Behrman et al., 1996).

Examination of 43 corpora lutea from non-pregnant cows representing four stages of the ovarian cycle showed that  $\beta$ -carotene and  $\alpha$ -tocopherol continuously increased from stage I to stage IV (Schweigert, 2003). Two other studies have independently reported that bovine corpus luteum  $\beta$ -carotene levels are positively correlated with plasma progesterone levels (Haliloglu et al., 2002; Rapoport et al., 1998). In rat ovary, follicular development was associated with an increase in vitamin A and luteal regression was associated with an increase in both vitamins A and E (Aten et al., 1992). Studies in bovine luteal cells in culture showed that  $\beta$ -carotene depletion caused inactivation of P450<sub>scc</sub> and its crosslinking to adrenodoxin. The addition of  $\beta$ -carotene at levels found in bovine serum, but not  $\alpha$ -tocopherol or ascorbic acid, inhibited the cross-linking (Young et al., 1995). The fact that both adrenodoxin and P450 are metal carrying proteins that generate free radicals during electron transfer make these proteins prone to metal-catalyzed oxidation and crosslinking reactions (Liu et al., 2004; Stadtman, 1993). The *in vitro* effect of  $\beta$ -carotene most probably reflects the function of the antioxidants to prevent oxidative damage *in situ* as well.

**Antioxidants in the testis.** The Leydig cells in the interstitium, located in between the seminiferous tubules of the testis, are the major source of androgenic steroids (Payne and Hales, 2004; Zirkin and Chen, 2000). The Leydig cells develop and reach their full steroidogenic potential after puberty (Cummings and Kavlock, 2004). With aging in males, the capacity for testosterone biosynthesis declines as a consequence of multiple alterations in the hypothalamic-pituitary axis and Leydig cell steroidogenic system (Wang and Stocco, 2005; Zirkin and Chen, 2000). As in other steroidogenic tissues, testis has the usual complement of antioxidant enzymes, including SOD, catalase, glutathione peroxidase, and glutathione transferase (Kukucka and Misra, 1993; Peltola et al., 1996) and

**Table 5** Antioxidant levels in steroidogenic tissues.

Antioxidant	Species	Tissue	Concentration*	Reference
Ascorbate	Rat	Adrenal	2.5–5 mg/g tissue	(Mitani et al., 2005)
$\alpha$ -tocopherol	Rat	Adrenal	30–50 $\mu$ g/g tissue	(Burczynski et al., 2001)
Ascorbate	Bovine	C. luteum	4–10 mg/g tissue	(Rapoport et al., 1998)
$\beta$ -carotene	Bovine	C. luteum	6–216 $\mu$ g/g tissue	(Arikan and Rodway, 2001) (Haliloglu et al., 2002)
$\alpha$ -tocopherol	Bovine	C. luteum	1–132 $\mu$ g/g tissue	(Rapoport et al., 1998) (Schweigert, 2003)
Vitamin A	Bovine	C. luteum	3.5–5.5 $\mu$ g/g tissue	(Haliloglu et al., 2002)

C. luteum: Corpus luteum.

\*Concentration per tissue wet weight.

small antioxidants (Chen et al., 2005; Livera et al., 2002). As in the ovary, stimulation of the testicular interstitial tissue increases the activities of antioxidant enzyme glutathione peroxidase (Peltola et al., 1996).

The findings of some studies provide evidence for the hypothesis that ROS produced as a by-product of steroidogenesis may be responsible for age-related decline in testosterone production by the Leydig cells (Chen and Zirkin, 1999; Zirkin and Chen, 2000). A method that has been used to test this hypothesis is the blockade of testicular steroidogenesis by administering a high dose of testosterone that suppresses LH secretion via a negative feedback mechanism on the hypothalamo-pituitary axis, and consequently inhibits Leydig cell testosterone production. In rats, testosterone treatment for 8 days decreased catalase, glutathione peroxidase, and glutathione transferase activities in the interstitial tissue (Peltola et al., 1996). A more recent study examined the effect of contraceptive doses of testosterone administered to male rats for 8 months using Silastic implants (Chen and Zirkin, 1999). Compared to control rats of the same age, the testosterone production capacity of the rats that had the long-term suppression was higher (Chen and Zirkin, 1999). The explanation provided for these findings was that the placement of the Leydig cells in a state of steroidogenic “hibernation” reduced steroidogenic activity and consequently decreased accompanying ROS production and prevented aging. Thus, after removal of the blockade, the testis started functioning at the levels observed for young rats.

Exogenously added  $H_2O_2$  was observed to inhibit both cAMP-stimulated progesterone production and StAR protein expression in MA-10 tumor Leydig cells, but did not effect P450<sub>scc</sub> enzyme levels (Diemer et al., 2003). These results suggest differential sensitivity of different parts of the steroidogenic machinery to ROS effects. Yet, the exogenous application of the  $H_2O_2$  also raises the question whether the differential effects may be a result of differential exposure to the damaging effects of the externally applied  $H_2O_2$ .

Similar to the findings in adrenocortical cells (see previous sections), culturing Leydig cells with vitamin E, or administering vitamin E to rats, showed protective effects on steroidogenic function (Chen et al., 2005).

## SUMMARY

In summary, the studies reviewed in this section showed the following associations between mitochondrial P450 systems and antioxidants present in their environment:

1. Increased activity or expression of adrenodoxin or mitochondrial P450s is associated with increased ROS production and oxidative damage.
2. Steroidogenic cells have a full spectrum of antioxidant molecules, which in some tissues reach the highest level in the body.
3. Steroidogenic capacity correlates with the levels of antioxidant enzymes. The expression of some antioxidant enzymes are activated by trophic hormones that stimulate steroidogenesis.
4. Depletion of specific antioxidants can lead to loss of activity of “sensitive” mitochondrial P450s.
5. Addition of antioxidants can prevent or reverse oxidative damage effects induced by the P450 system proteins.

It should be emphasized that these points apply for the specific cases reviewed, and do not represent general rules for all mitochondrial P450 and antioxidant combinations in different systems.

## ABBREVIATIONS

AdR	adrenodoxin reductase
ROS	reactive oxygen species
SOD	superoxide dismutase
Subscript “ <sub>red</sub> ”	reduced

## DEDICATION

This review is dedicated to the memory of my dear friend and colleague Dr. David Kupfer (Z.L) whose research career was devoted to the study of the role of cytochrome P450 type enzymes in drug metabolism (Schenkman, 2005; Stresser, 2006).

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