Impact of Aging on Steroid Hormone Biosynthesis and Secretion

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Abstract: Steroid hormones are lipophilic, low-molecular weight organic compounds all of which are derived from cholesterol. They are primarily synthesized by steroidogenic glands such as gonads (ovary and testis), the adrenal gland and, during pregnancy, by the placental trophoblasts. Limited steroid synthesis also takes place in the brain. Steroid hormones are crucial for the proper functioning of the body. They mediate a wide variety of vital physiological functions, ranging from maintaining normal reproductive function and secondary sexual characteristics, to regulating virtually every metabolic process in the body. Like many age-related endocrine disorders, aging also progressively impacts the tissue-specific synthesis and secretion of steroid hormones. The goal of this review is to summarize the effects of aging on steroid hormone synthesis and secretion by the adrenal gland and gonads of both human and experimental animal models, to describe the potential involvement of excessive oxidative stress in mediating age-related alterations in steroidogenesis, and to discuss the possible underlying mechanisms involved.

Keywords: Glucocorticoids, Mineralocorticoids, Androgens, Estrogens, Progestins, Gonadal Steroids, Cholesterol, Oxidative Stress, Cholesterol Transport Proteins, Antioxidants.

INTRODUCTION

The steroid hormones are lipophilic, low-molecular weight organic compounds all of which are derived from cholesterol [1-6]. They share a typical but not identical cyclopentano-perhydrophenanthrene nucleus (steroid nucleus) of cholesterol. They are mainly synthesized by steroidogenic glands such as gonads (ovary and testis), the adrenals and, during pregnancy, by the placental trophoblasts, and are then released into the bloodstream [1-7]. Based on their physiological functions, the steroid hormones are broadly classified into three categories: the glucocorticoids, mineralocorticoids and sex steroids (androgens and estrogens). The adrenal cortex produces mineralocorticoids (aldosterone, deoxycorticosterone), glucocorticoids (cortisol, corticosterone), and sex steroids (mainly the androgen precursors dehydroepiandrosterone [DHEA] and androstenedione); mineralocorticoids are mainly produced in the zona glomerulosa zone, whereas glucocorticoids are principally produced in the zona fasciculate of the adrenal cortex. The adrenal sex steroid (androgens) are mainly a product of the zona reticularis of the adrenal cortex [1-6,8].

Testosterone is a major androgen produced in the testis, and its primary site of synthesis is in Leydig cells [1-6,9]. The ovaries secrete three major sex steroids: estrogens, androgens and progestin [1-6,10]. The 17β-estradiol is the principal estrogen secreted by the ovarian theca cells. The ovary also secretes a variety of androgens including DHEA, androstenedione, testosterone, and dihydrotestosterone. They are produced by the theca cells and to a lesser degree by ovarian stroma cells. The major ovarian androgen, androstenedione is produced by the theca cells and transported to ovarian granulosa cells for estrogen production. Theca cells and to a lesser degree ovarian stroma cells also secrete a variety of other androgens including DHEA, testosterone, and dihydrotestosterone (DHT). The major progestins include pregnenolone, progesterone, and 17-hydroxyprogesterone. Among these, pregnenolone is produced by all steroidogenic tissues where it serves as a precursor for all steroid hormones (Fig. **1**). Progesterone is the principal secretory product of the corpus luteum and is responsible for the implantation of the fertilized ovum and maintenance of pregnancy during the first 6-8 weeks of gestation, but during pregnancy the main source for progesterone is the placenta [7]. It can also be converted to estrogen or testosterone in extraglandular tissues.

Steroid hormones are vital to the smooth functioning of a number of physiological functions (Table **1**). For example, female-sex steroids, estrogens and progestin are required for follicular development, ovulation, development, maturation and maintenance of secondary sex organs (female sexual determination), sexual behavior patterns, and also, for sustaining pregnancy when it occurs [7,11,12]. Estrogens also play an important role in cardiovascular physiology, bone integrity, neuronal growth and differentiation, neuroprotection, cognition, and behavior [13,14 and references therein]. Testosterone, the most prevalent male sex-hormone (androgen) and its biologically active form, DHT are necessary for normal spermatogenesis and development, maturation and function of secondary sex organs (male sexual determination) [12,15]. Beyond its reproductive function, testosterone is responsible for increased muscle mass, sexual function and libido, body hair and decreased risk of osteoporosis, and

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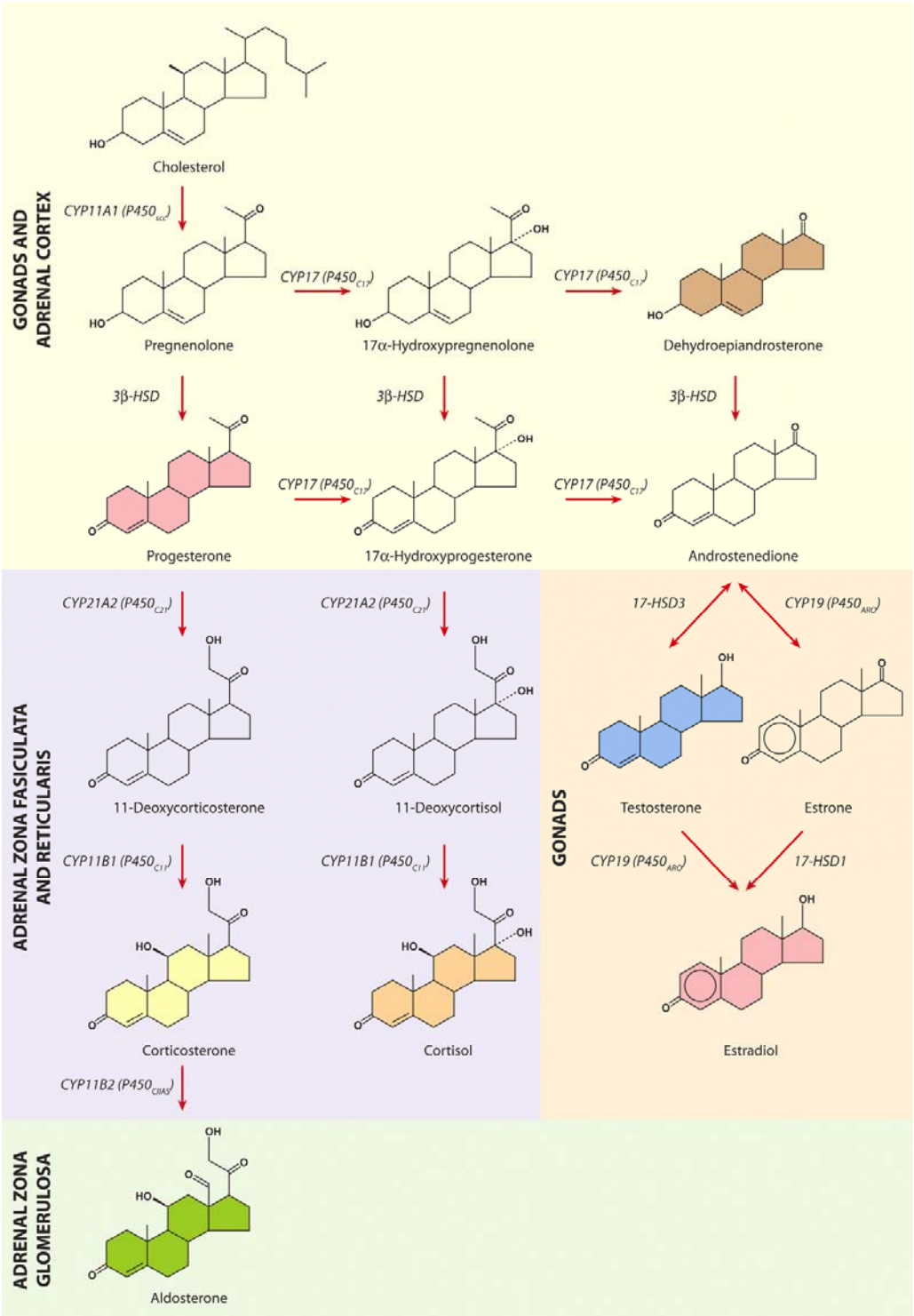

Fig. (1). Pathways of steroid biosynthesis in adrenal glands and gonads. Enzymatic reactions that are common to both adrenal and gonadal tissues, specific for adrenal (i.e., zona fasiculata and reticularis and zona glomerulosa) gland and gonadal tissues are shown shaded with different colors. Key enzymes are shown above the arrows indicating the specific enzymatic reactions. Modified from White [1], Conley and Bird [2], Payne and Hales [3] and Miller and Auchus [6].

also takes part in the nervous system [16,17]. Interestingly, a very recent study by Oury *et al.*, [17a] suggests that osteocalcin, a hormone produced by bone-building cells (osteoblasts) may have a role in regulating male fertility. These investigators, in a series of experiments using mice engineered to generate only low levels of osteocalcin demonstrated that male mice had lowered fertility due to reduced testosterone production. This effect appears to be specific for testis since osteocalcin levels failed to influence ovarian estrogen production and no impact on the fertility of female mice.

Table 1. Physiological Functions of the Major Steroid Hormones

Steroid Hormone	Functions
Progesterone (Progestin)	Prepares uterus lining for implantation of ovum and mammary glands for lactation
Cortisol (Glucocorticoid)	Helps control carbohydrate, protein and lipid metabolism; promotes gluconeogenesis; favors breakdown of fat and protein (fuel mobilization); exerts anti-inflammatory actions; protects against stress; reduces immune responses; helps to maintain blood pressure through its hypertensive actions.
Aldosterone (Mineralocorticoid)	Helps to regulate body's water and electrolyte balance. Maintains blood volume and blood pressure by increasing sodium reabsorption in exchange for K^+ and H^+ at electrolyte-transforming epithelia of the kidney.
Dehydroepiandrosterone (DHEA) and Dehydroepiandrosterone sulfate (DHEA-S) (Adrenal androgens)	The sulfated form of DHEA, DHEA-S is suggested to affect a wide variety of physiological systems including neurological, immune, aging and somatic growth and development. DHEA and DHEA-S are an important source of testosterone and estrogen in peripheral tissues, contributing as much as 50% of total peripheral androgens in men, 75% of peripheral estrogen in premenopausal women, and 100% of peripheral estrogen in postmenopausal women.
Testosterone (Androgen)	Controls the development of male secondary sex characteristics; regulates sperm production; promotes increased muscle mass through increased protein formation; prevents bone resorption
Estradiol (Estrogen)	Controls the development of female secondary sex characteristics; regulates menstrual cycle; contributes to the hormonal regulation of pregnancy and lactation; prevents bone resorption

The adrenal mineralocorticoid, aldosterone, maintains salt and water balance, and hence blood pressure [18], while the adrenal glucocorticoid, cortisol (corticosterone in rodents), is essential for diverse biological functions including regulation of carbohydrate, protein, and lipid metabolism in a manner nearly opposite to that of insulin, stress adaptation, inflammatory reactions, the immune system and stress adaptation [19]. The functional roles of adrenal androgens are not well understood, but considerable evidence now indicates that they are involved in the regulation of bone mineral density, muscle mass, well-being, and libido, as well as beneficial effects against skin atrophy, type 2 diabetes, and obesity [20].

Like many age-related endocrine disorders, aging also progressively impacts the tissue-specific synthesis and secretion of steroid hormones [21-24]. The goal of this review is to summarize the effects of aging on steroid hormone synthesis and secretion by the adrenal gland and gonads of both human and experimental animal models, describe the potential involvement of excessive oxidative stress in mediating age-related alterations in steroidogenesis, and discuss the possible underlying mechanisms involved.

STEROIDOGENESIS: AN OVERVIEW

Although the focus of this review is the age-related alternations in steroid hormone synthesis and secretion in the adrenal gland and gonads, in order to familiarize the readers about the subject, first, a brief overview regarding the critical events involved in steroid hormone biosynthesis is presented.

Source of Cholesterol

Substrate

Cholesterol is an obligate intermediate in the synthesis of all of the steroids produced by the adrenal, ovary, testis and placenta. Cholesterol can be obtained from three principal sources: a) cholesterol synthesized *de novo* from the acetate; b) cholesterol derived from the circulating lipoproteins; and c) cholesterol recruited by the hydrolysis of cytoplasmic cholesteryl esters stored in the form of lipid droplets (Fig. **2**) [25,26]. In some cultured cells including steroidogenic cells, cholesterol can also be obtained from the plasma membrane [27-29].

Low-density Lipoprotein (LDL) Receptor (LDL-R)-mediated Endocytic Uptake of Apolipoprotein B (apoB) or Apoe containing Plasma

Lipoproteins

The principal source of cholesterol utilized by the human adrenal and ovary is provided from the circulation in the form of low-density lipoprotein (LDL)-cholesterol. The first step in acquiring LDL-cholesterol is the binding of apolipoprotein B-100 (apoB) cholesterol-rich LDL particles to the cell surface of LDL receptors [30]. LDL is then internalized *via* the receptor-mediated endocytosis, and a drop in the pH (from ~7.0 to ~5.0) causes the LDL to separate from its receptor. The vesicle then pinches apart into two smaller vesicles: one containing free LDLs; the other containing now-empty receptors. The vesicles with LDL particles fuse with lysosomes and free cholesterol produced following hydrolysis by enzymes of the lysosome which is released into the

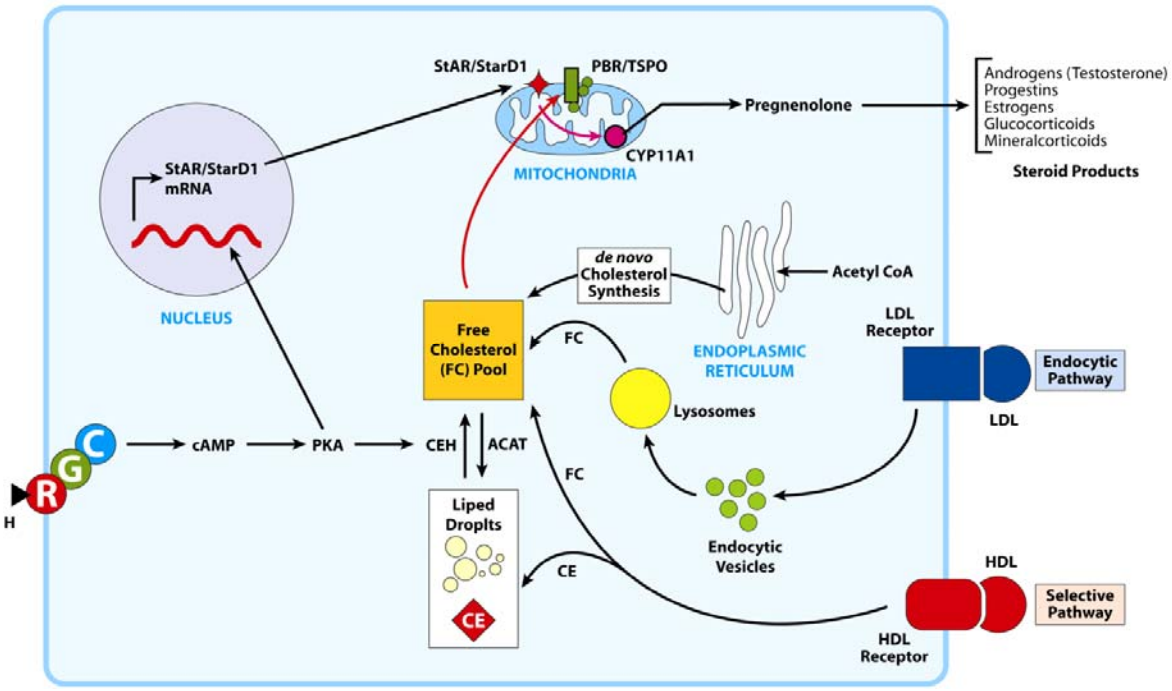

Fig. (2). Cholesterol metabolism for the biosynthesis of adrenal and gonadal steroids. LDL cholesteryl esters (CEs) or apoE containing HDL (apoE-HDL) CEs can be internalized *via* the LDL-receptor (LDL-R)-mediated endocytic pathway, whereas LDL and HDL CEs can be 'selectively' delivered into the cell *via* the scavenger receptor class B, type I (SR-BI)-mediated selective patrhway. CEs delivered by LDL-R-endocytic are hydrolyzed to free (unsterified) cholesterol (FC), whereas selectively delivered CEs are hydrolyzed by neutral cholesteryl ester hydrolase (CEH) (hormone-sensitive lipase, HSL). FC can also be derived from the the stored CEs in the form of lipid droplets through the actions of CEH or *via* the de novo synthesis from the acetyl CoA. The newly acquired FC is transported into mitochondria *via* combined actions of steroidogenic acute regulatory protein (StAR/StarD1) peripheral-type benzodiazepine receptor/transporter protein (PBR/TSPO) for metabolism by cholesterol side-chain cleavage enzyme (P450scc/CYP11A1). Any excess FC cholesterol is esterified for storage by acetyl CoA: cholesterol acyltransferase (ACAT). C, adenylate cuclase; G, G proteins (guanine nucleotide-binding proteins); H, tropic hormones, ACTH, FSH and LH/hCG; R, Tropic hormone (LH/hCG, FSH, ACTH) receptor.

cytosol [30-32]. The released cholesterol is either used directly for product formation (e.g., membrane biogenesis, steroid synthesis) or stored as cholesterol esters in the form of lipid droplets. The vesicles with empty receptors fuse with plasma membrane and deliver the LDL receptors to the cell surface for reuse. In addition to LDL, other plasma lipoproteins containing apoE (e.g., apoE-enriched rat or mouse high-density lipoprotein [HDL] are also processed *via* this endocytic pathway).

Scavenger Receptor Class B, Type I (SR-BI)-mediated 'Selective' Uptake of the Cholesteryl Esters by Adrenal Gland and Ovary

In contrast to humans, in the rodent adrenal and ovary, apoE-poor HDL-cholesterol is the preferential source of cholesterol utilized for steroidogenesis. The uptake of HDL cholesterol by steroidogenic cells, however, involves a unique pathway, termed 'selective' pathway in which cholesteryl esters (CEs) are selectively transferred to the cell interior without the parallel uptake and degradation of the HDL particle itself (Fig. **2**) [33,34]. SR-BI, a multiligand cell surface receptor, binds HDL with high affinity and mediates selective cholesterol uptake [33,34]. The selectively delivered CEs are either hydrolyzed by the neutral cholesteryl ester hydrolase (CEH) and released as cholesterol used for steroiodogenesis, or stored in cellular lipid droplets [33,35].

Cholesterol Requirement of Testicular Leydig Cell

The requirement for endogenously synthesized *vs.* plasma lipoprotein-derived cholesterol for testosterone synthesis in testicular Leydig cells also varies according to the species. For example, in the rodent (rat) Leydig cells, the cholesterol that is synthesized de novo within the cell is the most important source of testosterone synthesis [36-39], but in the human testis, both endogenously synthesized cholesterol as well as LDL-derived cholesterol contribute to testosterone production [39,40]. As noted above, all steroidogenic cells, however, contain the intracellular cholesterol pool in the form of cholesterol esters (lipid droplets) which are regarded as a short-term store of substrates that enables cells to respond rapidly to trophic hormone stimulation [25,33,39].

Hormonal Regulation of Steroidogenesis

The type of a particular steroid hormone synthesized by a given steroidogenic cell type depends upon its sensitivity to a particular type of tropic hormone (Tables **2** and **3**) and its genetically expressed complement of steroidogenic enzymes (Fig. **1**). Thus, adrenocorticotropic hormone (ACTH) stimulates glucocorticoid (cortisol/corticosterone) in adrenocortical fasiculata-reticularis cells [41,42]; ACTH or angiotensin II increases mineralocorticoid, aldosterone synthesis in adrenal glomerulosa cells [43,44]; human chorionic gonadotropin

Table 2. Major Steroid Synthesized by the Steroidogenic Cells of Adrenal, Ovary and Testis

Steroidogenic Cells	Regulatory Hormone	Steroid Products
Adrenal gland		
Zona glomerulosa cells	Angiotensin II, K^+ and to lesser extent ACTH	Aldosterone (Mineralocorticoid)
Zona fasciculate cells	ACTH	Corticosterone*, Cortisol¶ (Glucocorticoids)
Zona reticularis cells	ACTH, pro-opiomelanocortin- derived peptides, other factors	Androstenedione, Dehydroepiandrosterone (DHEA) and Dehydroepiandrosterone sulfate (DHEA-S) (adrenal androgens)
Ovary		
Granulosa cells	FSH	Progersterone (progestin) Estradiol (estrogen)
Luteinized granulosa cells	LH	Progesterone (progestin)
Luteal cell	LH	Progesterone (progestin)
Theca-interstitial cells	LH	Androsterone (androgen)
Testis		
Leydig cells	LH	Testosterone (androgen)

*Corticosterone is synthesized and secreted by the rodent adrenal gland. ¶Cortisol is the sole glucocorticoid secreted by the human adrenal gland. ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone

Table 3. Major Enzymes Involved in the Biosynthesis of Adrenal and Gonadal Steroids

Common Name	Old Designation	Current Designation
Cholesterol-side chain cleavage enzyme; desmolase	$P450_{scc}$	**CYP11A1**
3β-Hydroxysteroid dehydrogenase	3β-HSD	**3β-HSD**
17 α-Hydroxylase/17,20 lyase	$P450_{c17}$	**CYP17**
21-Hydroxylase	$P450_{C21}$	**CYP21A2**
11β-Hydroxylase	$P450_{C11}$	**CYP11B1**
Aldosterone synthase	$P450_{C11AS}$	**CYP11B2**
Aromatase	$P450_{aro}$	**CYP19**

(hCG) or luteinizing hormone (LH) promotes progesterone synthesis in luteinized granulosa-luteal cells [45,46]; follicle-stimulating hormone (FSH) stimulates progesterone and estrogen synthesis in granulosa cells [47,48]; and LH regulates androgen production both in theca-interstitial cells (androsterone) [49,50] and testicular Leydig cells (testosterone) [51,52].

The first reaction in the conversion of cholesterol substrate (C_{27}) to C_{21} (pregnenolone, progesterone, glucocorticoids and mineralocorticoids), C_{19} (androgens), and C_{18} (estrogens) steroids involves the cleavage of a 6-carbon unit from 27 carbon cholesterol to form the common steroid precursor, 21carbon pregnenolone [2,3,6] (Fig. **1**). This NADPH dependent reaction is catalyzed by the side-chain cleavage (SCC) cytochrome P450 (P450scc or CYP11A1) complex, which is comprised of a flavoprotein (NADH-adrenodoxin reductase), a Fe_2-S_2* type iron-sulfur protein (adrenodoxin) and a hemoprotein CYP11A1 (P450scc) localized in the inner mitochondrial membranes of steroid producing cells [2,3,6].

This initial reaction is the principal committed, regulated, and rate-limiting step in steroid biosynthesis. The rate limiting nature of this step is not determined by the activity of CYP11A1 (i.e., enzymatic conversion of cholesterol to pregnenolone), but rather delivery of cholesterol to the substrate site of CYP11A1 (translocation of cholesterol from outer to an inner mitochondrial [steroidogenic] pool readily accessible to CYP11A1) [53]. The tropic hormones, ACTH and gonadotropins (LH, FSH) acutely stimulate this process by facilitating the availability of cytosolic free cholesterol and its transport to and accumulation in the inner mitochondrial CYP11A1 sites. When steroidogenic cells are stimu-

lated with tropic hormones in the presence of protein synthesis inhibitors such as cycloheximide, cholesterol accumulates in the outer mitochondrial membrane and is not transferred to the inner mitochondrial membrane [54-57]. Therefore, it has been proposed that a *labile* protein is required for cholesterol delivery to the inner mitochondrial membrane microdomains containing CYP11A1 [26,53,59].

The entire process of intracellular cholesterol transport to mitochondria can be broadly divided into two separate but equally important steps. In the first step, the tropic hormone-mediated increased cAMP formation (second messenger) stimulates PKA [54,60], leading to phosphorylation (activation) of a cholesteryl esterase, which, in turn, hydrolyzes stored cholesteryl esters (in the form of lipid droplets) and generates increased concentrations of free cholesterol, the substrate for CYP11A1 [26,35]. Subsequently, the mobilized cholesterol is transported to the outer mitochondrial membrane. In addition, depending on cell type, the cAMP-PKA signaling cascade may also directly mobilize cholesterol from the plasma membrane or other cellular membranes to the outer mitochondrial membrane [27-29]. Because cholesterol is a hydrophobic molecule and diffuses poorly in an aqueous environment, a number of putative factors including cholesterol transport proteins such as sterol carrier protein$_2$ (SCP_2), steroidogenesis activator polypeptide (SAP), cytoskeleton components/structures, StarD proteins (see below) and changes in cellular architecture have been suggested to facilitate cholesterol transport to the outer mitochondrial membrane, however, their mechanisms of action have not been completely known [58].

The second critical step is the delivery of the cholesterol substrate to inner mitochondrial membrane sites of CYP11A1 [53,56,57]. As noted above, this step is considered rate-limiting because hydrophobic cholesterol cannot rapidly diffuse through the aqueous intermembrane space of the mitochondria to support acute steroid synthesis and requires the participation of a *de novo* synthesized labile protein [61-65]. The search for the putative regulatory protein identified several candidate proteins, namely SCP2, DBI, SAP, peripheral benzodiazepine receptor (PBR), steroidogenesis inducing protein (SIP), and steroidogenic acute regulatory protein (StAR) [58].

Among them, the StAR protein possesses all the necessary characteristics of the acute regulator of steroid hormone biosynthesis in steroidogenic cells [58,66], i.e., it is a synthesis specifically induced in the adrenal and gonads in response to tropic hormonal stimulation, is highly labile, and its expression is sensitive to the protein synthesis inhibitor, cycloheximide [58,67]. This candidate protein was initially described by Orme-Johnson and colleagues [64,65]. The discovery of mutations in the StAR gene in patients with lipid congenital adrenal hyperplasia was critical to the elucidation of the role of StAR protein in the acute steroidogenic response [68]. Lipoid congenital adrenal hyperplasia patients have markedly impaired gonadal and adrenal steroidogenesis [due to an inability to efficiently transport into the mitochondria] associated with a massive accumulation of cholesterol in lipid droplets] [68]. Deletion of the murine *StAR* gene by the homologous recombination yielded an identical phenotype of impaired steroidogenesis and lipid accumulation in the adrenal gland, and to a lesser degree in testicular Leydig cells and none in the ovary of StAR knockout mice [69]. StAR is synthesized as a short-lived cytoplasmic 37-kDa protein with a mitochondrial leader peptide that is cleaved upon mitochondrial import to yield the long-lived intramitochondrial 30-kDa form [58]. It is now well-established that StAR functions as a sterol transfer protein [70], binds cholesterol [71,72], mediates the acute steroidogenic response by moving cholesterol from the outer to the inner mitochondrial membrane [73], acts on the outer mitochondrial membrane [73-75], and requires the structural change previously described as a pH-dependent molten globule [76]. StAR is also a prototype of a family of proteins that contain START (StAR-related lipid transfer) domains (StarD proteins) [77], of which StarD3/MLN64, StarD4, StarD5 and StarD6 exhibit steroidogenic potential [78,79].

Peripheral-type benzodiazepine receptor (PBR), recently renamed mitochondrial translocator (TSPO) has also been implicated in the transport of cholesterol across mitochondrial membranes in steroidogenic cells [80,81]. PBR is expressed ubiquitously on the OMM, but is most abundant in steroidogenic cells [80]. PBR ligands stimulate steroidogenesis and promote translocation of cholesterol from OMM to the IMM in testicular Leydig cells, ovarian granulose cells, and adrenocortical cells [81-85]. Targeted disruption of the PBR/TSPO gene in rat Leydig R2C cells (PBR-deficient cells) blocked the cholesterol import into the mitochondria and dramatically reduced steroid production, whereas reintroduction of PBR/TSPO in this cell line restored steroidogenesis [86,87]. Likewise, mutation of a single amino acid residue in the "cholesterol recognition amino acid consensus" domain in the carboxyl-terminal region disrupts cholesterol binding and transfer to IMM [76,87]. PBR/TSPO is a component of the multimeric 140-200-kDa complex located on the OMM especially at the OMM-IMM contact sites. The complex consists of 18-kDa PBR or TSPO itself (and its polymorphic forms), the 34 kDa voltage-dependent anion channel (VDAC), the 30-kDa adenine nucleotide translocator (ANC), a 10-kDa protein (pk 10), PBR-associated protein 1 (PRAX-1), and the PBR and protein kinase A (PKA) regulatory subunit RIα-associated protein (PAP7) [88].

Multiple lines of evidence now indicate that PBR and StAR are likely to work in concert in mediating the movement of cholesterol from OMM to IMM. Although a physical interaction between StAR and PBR/TSPO has not been established, FRET measurements indicated that StAR/StarD1 and PBR/TSPO come within the 100 A^o of each other [89]. Moreover, Hauet *et al* [87] reported that isolated mitochondria from mouse Leydig MA-10 cells that express the Tom20/StAR fusion construct produce steroids at a maximal level, but if the cells are treated with PBR-antisense oligonucleotides, their ability to synthesize the steroid is lost; on the contrary, re-introduction of recombinant PBR into the mitochondria *in vitro* rescued the steroidogenesis [87,90]. Liu *et al.,* [91] provided evidence that hormonal stimulation of a Leydig cell line leads to formation of a StAR/StarD1-PKARIα-PAP7-PBR/TSPO macromolecular signaling complex on the outer mitochondrial membrane that mediates the effect of hormones on mitochondrial cholesterol transport and steroidogenesis. Recently, evidence is presented to suggest that StAR interacts with VDAC1, and

with phosphate carrier protein (PCP) on the OMM to initiate the action of StAR [92]. These studies further point to a functional cooperation between the PBR/TSPO and StAR/StarD1 proteins and possibly participation of many other proteins as well [76,88,90,93,94].

Steroidogenic Steps and Enzymes Involved in Biosynthesis of Steroids in a Tissue-specific Manner

Following cholesterol transport to mitochondria, it is cleaved by the cytochrome P-450 side-chain cleavage ((P450scc; CYP11A1), adrenodoxin, and flavoprotein system to generate pregnenolone [2,3,6]. Pregnenolone is then shuttled from the mitochondria to the smooth endoplasmic reticulum where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). Depending on cell type, the pregnenolone and progesterone are further metabolized to sex steroids or corticosteroids (Fig. **1**). In testicular Leydig cells, the synthesis of testosterone can occur through two biosynthetic pathways: the Δ^{-5} pathway and Δ^{-4} pathway. In man the major pathway of biosynthesis is *via* Δ^{-5} pathway including pregnenolone, 17-hydroxypregnenolone, and androstenediol. In other species, testosterone biosynthesis proceeds *via* the Δ^{-4} pathway including progesterone, 17-hydroxyprogesterone, and androstenedione.

The principal steroid-producing cells of the ovary, namely the granulosa, theca and corpus luteum cells, possess the complete enzymatic complement required for steroid hormone formation. The main pathway of steroid hormone synthesis in the human corpus luteum is the Δ^{-4} pathway which involves the conversion of pregnenolone to progesterone. In the human ovarian follicle, the Δ^{-5} pathway is the preferred pathway for the formation of androgens and estrogens, because theca cells of the human ovary metabolize 17-hydroxypregnenolone more efficiently than 17-hydroxyprogesterone. However, the predominant steroid differs among each of these cell types so that the corpus luteum primarily forms progesterone and 17-hydroxy progesterone in response to LH, the theca and stromal cells secrete androgen, and the granulosa cells secrete estrogen predominantly. LH regulates the first step in steroid hormone biosynthesis by controlling the conversion of cholesterol to pregnenolone and its subsequent metabolism to androgens in theca cells, whereas FSH (possibly plus LH) controls conversion of androgens to estrogens in granulose cells.

In zona fasiculata cells of the adrenal cortex, progesterone is hydroxylated to 17α-hydroxyprogesterone by CYP17 which is subsequently metabolized to 11-deoxycortisol (or deoxycorticosterone by CYP21A2. The final step in cortisol biosynthesis takes place in the mitochondria and involves the conversion of 11-deoxycortisol to cortisol by the enzyme CYP11B1. The C17,20 lypase activity associated with CYP17 of zona fasciculate and zona reticularis cells catalyzes the production of androgens, dehydroepiandrosterone (DHEA) and androstenedione. In the zona glomerulosa cells, progesterone is 21-hydroxylated by the enzyme CYP21A2 to yield 11-deoxycorticosterone. The next two steps in aldosterone biosynthesis are catalyzed by aldosterone synthase (CYP11B2) which converts 11-deoxycorticosterone to corticosterone and subsequently to aldosterone [2,3,6].

AGING AND TESTOSTERONE SECRETION

Humans

It is well-recognized as men age, plasma testosterone concentrations decline gradually after age 40, albeit with considerable variability between individuals (~0.4 to 2.6% per year) [95,96]. As early as 50-60 years ago it was realized that aging is accompanied by significant reduction in testosterone levels [97,98]. Although some follow-up studies failed to detect age-related decline in plasma testosterone levels in older men [99-104], subsequent population-based cross-sectional and longitudinal studies have confirmed progressive loss of testosterone with aging in healthy men [96,105-122]. Mirroring this decline in plasma testosterone concentration is an age associated increase in sex-hormone-binding globulin (SHBG) level [117], a major plasma carrier of testosterone, resulting in even more dramatic decreases in unbound free testosterone [110,123-125], and weakly bound bioavailable testosterone [126,127]. [In healthy male adults, circulating testosterone exists in three forms: testosterone strongly bound to SHBG, testosterone weakly bound to albumin and free testosterone]. Approximately 80% of the plasma testosterone is bound to SHBG and is not bioavailable. The remaining 20% is biologically active and consists of both weakly albumin-bound and free testosterone. According to one study, the Longitudinal Massachusetts Male Aging Study, total plasma testosterone decreases at a rate of 1.6% per year, while bioavailable testosterone decreases at a rate of 2-3% per year [117]. In contrast, levels of SBG increase 1.2% per year. The overall impact of these changes is not only a significant decrease in all three subfractions of testosterone, but also a significant shift toward functionally inactive bound testosterone as compared to bioavailable free testosterone.

Decreased testosterone availability in aging has been associated with parallel age-related decline in bone density, muscle mass, muscle strength, physical function, and sexual function [96,120-122,124,128]. Epidemiological studies have also correlated hypoandrogenemia with impaired quality of life, frailty, depressive mood and subtle impairments in cognitive function [122,124,125,128]. More recently, low-circulating testosterone has been linked to age-related metabolic abnormalities including body-wide reductions in rates of protein synthesis, abdominal obesity, diabetes, prediabetic states (insulin resistance, impaired glucose tolerance and metabolic syndrome), dyslipidiemia (alterations in LDL and HDL and plasma triglycerides) and increased risk of cardiovascular disease [125,129]. Although the precise causes have not been clarified, lower testosterone levels in the elderly male population is likely to result from defects in the hypothalamus, pituitary gland and/or testis [96,120,130-132]. The age-related decreases in testosterone secretion have been related to structure and function of Leydig cells. Several potential mechanisms for the loss of Leydig cell function has been proposed including: a) a reduction in Leydig cell number; b) a normal number of cells, each having defects in one or more enzymatic steps involving testosterone biosynthesis; c) a normal number of cells with reduced responsiveness to trophic hormone (luteinizing hormone or LH); and d) Leydig cell degeneration and dissolution [130,133].

There have been many reports linking age-related alteration in testosterone levels with the number and morphology of human Leydig cells. Sarjent and McDonald were the first to report significant reduction in Leydig cell number in autopsy samples of men with an age greater than 20 years [134]. Decreased Leydig cell numbers have also been reported by Tillenger [135], Frick [136], Harbitz [137] and Kaler and Neaves [138]. In contrast to these findings, Sniffen, [139], as well as Sokal [140] observed no loss of Leydig cells with age, while Kothari and Gupta [141] reported total Leydig cell mass to be increased in testes from their older men. Besides, a number of studies have also shown impaired testicular responsiveness to hCG stimulation [103,104,133,143-146] or to recombinant human LH [147,148] stimulation in aging men. Harman and Tsitouras [103], Nieschlag *et al.,* [104], Rubens *et al.,* [144], Nieschlag *et al.,* [142], and Nankin *et al.,* [146] all noted decreases in both the absolute and relative (i.e., ratio of stimulated to basal) testosterone responsiveness in elderly as compared to young men.

Relatively less information is available about the potential causes of the reduction in Leydig cell number and their responsiveness to LH/hCG stimulation during aging. The work of Sasano and Sadatoshi [149] and Takizawa and Hatakeyama [150] has demonstrated that changes in the microvasculature of the testis may be indirectly responsible for degenerative changes seen in the Leydig cells of older individuals. Several studies have provided evidence that loss of Leydig cell population in the adult human testis as a function of increasing age is caused by Leydig cell hyperplasia, dedifferentiation, degeneration and dissolution [151-153]. In addition, there is evidence for an impaired hypothalamic GnRH outflow and decreased pulse generation of GnRH, with pulses being generated more irregularly, normal or enhanced LH secretion when stimulated by exogenous GnRH pulses, reduced pulsatile LH-stimulated testosterone synthesis, and decreased negative feedback in older men [131,132,154-156]. From the above discussion, it is quite apparent that multiple alterations in hypothalamic-pituitary-testicular axis lead to the age-related decline in testosterone synthesis and secretion. In addition, with aging, the normal circadian rhythm of testosterone secretion is lost [132,133].

Experimental Animals

Use of experimental animals to study the effects of aging on Leydig cell steroidogenesis is advantageous in terms of the flexibility with which experiments can be designed and the availability of tissues and cells for more mechanistic studies. However, it should be pointed out that experimental findings derived from laboratory animals may not be entirely applicable to the human situation, since it appears that the patterns of hormone changes vary considerably from species to species and even from strain to strain. The rat is the most popular laboratory animal to examine the effects of aging on testicular androgen synthesis and secretion. A number of studies carried out during the past few decades have led to a clear demonstration that aging leads to significant reduction in blood testosterone levels in several rat strains [157-169].

One study reported both a reduction in circulating testosterone levels and loss of diurnal rhythm for testosterone in the older rats [170]. Similar to the age-related loss of basal blood testosterone concentrations, the acute response (<1-4h) of the rat testis to gonadotropin (hCG) stimulation *in vivo* is also impaired in older rats [171-173]. Moreover, chronic hCG treatment (3-7 days) was shown to restore plasma testosterone levels of older rats to levels comparable to those of similarly stimulated young mature Wistar [173] or Long-Evans [174] rats. In contrast, treating rats for up to three weeks with hCG, Lin *et al.,* [175] were unable to restore testosterone levels in 24-month old Sprague-Dawley rats to values seen in identically treated young mature rats.

There is no consensus whether or not aging also affects the quantity, structure or the organelle content of testicular Leydig cells. A number of studies indicate that the number of Leydig cells per testis is either unchanged or increased [162,176-178]. In contrast, occurrence of Leydig cell hyperplasia as a consequence of aging has also been reported in stallions [179] and humans [141]. Other studies suggest that Leydig cells undergo atrophic changes in size [168,169,176,180] and organelle content [178] with aging. Bethea and Walker [181] provided evidence showing that aging leads to decreased Leydig cell mass, at least in testis of Fischer rats.

When specifically challenged *in vitro*, isolated Leydig cells of older rats of several different strains show significantly less steroidogenic response to tropic hormone stimulation than do cells from young animals [162,175,178,181-183]. Only studies conducted by Kaler and Neaves [177] reported no significant differences in testosterone production by isolated Leydig cells from old rats as compared to similarly isolated cells from young controls [177]. Tsitouras *et al.,* [184] reported that *in vivo* pretreatment with, but not *in vitro* exposure to, hCG reverses the testosterone secretory defect of Leydig cells from old rats. Overall, these changes appear not to be a function of gonadotropin receptor activity, or cAMP formation [162,177,182,183,185,186] or a defect in the steroid hormone synthesizing enzymes [176,186] although there are some exceptions to this. For example, Pirke *et al.,* [162] reported significant loss of hCG binding to whole testicular membranes of old rats, while Tsitouras *et al.,* [182] observed only a modest decrease in hCG binding to membrane preparations from the purified Leydig cells. Likewise, aged Leydig cells from Brown Norway rats also exhibited a significant reduction in gonadotropin (LH) receptor content [187]. Contrary to these findings, other investigators failed to observe any reduction in gonadotropin binding either *in vivo* [162] or *in vitro* [177,185,188]. In addition, gonadotropin stimulation of cAMP production and PKA activity in Leydig cells was unaffected by the aging process [182,183,186]. Two reports, however, have provided evidence suggesting that aging negatively impacts on cAMP production and cAMP-stimulated PKA activity in Leydig cells and that such reduction in the cAMP signaling cascade may be functionally linked to decreased testosterone synthesis and secretion seen with advancing age [189,190]. On the other hand, studies from our laboratory indicate that testosterone secretory response to LH (endogenous gonadotropin), cholera toxin (a nonspecific stimulator of adenylate cyclase), forskolin (stimulator of adenylate cyclase) and cAMP agonists (e.g., Bt_2cAMP, 8Br-cAMP, 8CPT-cAMP), like hCG, is reduced by 60-70% in purified Leydig cells from older

animals [183]. These latter studies and others [184,186] strongly support the possibility that the 'aging' defect in the Leydig cells occurs beyond the hormone receptor binding and hormone-stimulated cAMP signaling cascade.

There is some evidence that aging affects the functional expression of certain steroidogenic enzymes involved in the conversion of cholesterol to testosterone in Leydig cells. Total testicular Δ^5-3β-hydroxysteroid dehydrogenase /Δ^5-Δ^4-isomerase (3β-HSD) activity, which converts pregnenolone → progesterone, 17α-hydroxypregnenolone → 17α-hydroxyprogesterone and dehydroepiandrosterone → androstenedione (Fig. **2**), has been reported to be decreased in old Long Evans rats, and this reduced activity was fully restored following hCG treatment of old animals *in vivo* [191]. Extensive studies carried out by Zirkin and colleagues [188,191,192] further demonstrated that the activity of CYP11A1 (P450scc), which catalyzes the conversion of cholesterol to pregnenolone, as well as 3β-HSD, 17-KSR and individual activities of CYP17 ($P450_{C17}$), namely 17α-hydroxylase and C17-20 lypase activities, which mainly convert progesterone to 17α-hydroxyprogesterone and androstenedione, respectively, decrease with aging. Western blot analyses revealed age-related changes in CYP11A1, 3β-HSD and CYP17 that were consistent with enzymatic activity analyses [188,190,192]. In contrast, no changes in testicular 3β-HSD were noted by Lehmann *et al* [176].

Chan *et al.,* [193] made an interesting observation that when dissected testes of old Long-Evans rats were incubated with [^{3}H]progesterone, they generated less testosterone and 5α-androstene-3α,17β-diol but produced similar amounts of 17α-hydroxyprogesterone, androstenedione, and 5α-dihdrotestosterone (DHT) and large amounts of an inactive metabolite of testosterone, 7α-hydroxyprogesterone, as compared to young rat testis. From these observations, the authors concluded that a shift towards increased formation of 7α-hydroxyprogesterone is responsible for lower testosterone production in old rats. Liao *et al.,* [183] reported that the *in vitro* defect of testosterone production in old Leydig cells is not observed when an incubation medium is supplemented with freely-diffusible steroid precursors, 25-hydroxycholesterol, 20α-hydroxycholesterol or 22(R) hydroxycholesterol, i.e., aging showed no inhibitory effect on hydroxycholesterol-supported steroidogenesis. These results were interpreted to suggest that aging interferes with the events connected with the intracellular cholesterol mobilization and its transport to mitochondria [183]. Indeed, it has been shown by the same authors that aging directly affects LH-mediated cholesterol transfer into and within the mitochondria [183]. This conclusion is further supported by the observation that significant decreases occur in the expression of StAR/StarD1 [194,195] and PBR/TSPO [196,197] proteins, which are now recognized as crucial players in facilitating cholesterol transport to mitochondria for steroidogenesis.

The impact of aging on testicular steroidogenesis has also been studied in mice, but not as extensively as in rats. Eleftheriou and Lucas [198] and Nelson *et al.,* [199] reported that plasma testosterone levels were similar in both young and old mice. In contrast, Bronson and Desjardins [200] and Coquelin and Desjardins [201] all observed lower levels of plasma testosterone, LH, but not FSH in mice of 18-30 months of age. Likewise, a recent study reported that the plasma testosterone and androstenedione levels in old mice (18-19 months of age) on a heterogeneous genetic background derived from the Ola-BALB/cJ, C57BL/6J, and C3H/J strains, were lower as compared to young mice (3-4 months of age) of a similar genetic background [202]. In addition, plasma testosterone responses to LH stimulation were attenuated in old mice, whereas plasma androstenedione responses were insensitive to aging [202]. Given these conflicting reports, obviously more studies are needed to firmly establish the effects of aging on testosterone production in mice.

AGING AND SECRETION OF FEMALE SEX-STEROIDS

Substantial and important age-related changes also occur in the ovary leading to a gradual decline in the reproductive potential of women with advancing age. Undoubtedly, the most widely recognized aging effect on the reproductive system in women is the age-associated transition from the normal cycling ovary to perimenopause to the menopause state. By the mid-sixth decade of life, all women undergo menopause, which is characterized by the permanent cessation of menstruation resulting from exhaustion of the ovarian follicular reserve as well as loss of their activity. Ovarian follicles are the source of fertilizable ova for reproduction, and are also responsible for biosynthesis of sex steroids, progestin, androgens and estrogens, all of which are essential for normal reproductive function and maintenance of fertility. Although age-associated depletion of ovarian follicles is the ultimate cause of menopause and end of reproductive function in females, concomitant hormone changes including sex steroids also contribute significantly to ovarian aging.

The purpose of this section is to summarize our current knowledge about the age-related alterations in human ovarian steroid hormone synthesis and their relevance to menopause. Although significant information is also currently available about the impact of aging on ovarian steroidogenesis using experimental animal models [203-211], to conserve space and given the fact that physiological events connected with the reproductive cyclicity and ovarian sex steroid synthesis in animals, particularly rodents, are significantly different from that of humans, we will restrict our discussion to the aging human ovary only. Also, although aging is known to indirectly influence the ovarian steroid hormone production through modulation of the hypothalamic-pituitary-ovarian axis, again, due to space constraints, this aspect of ovarian regulation will not be discussed here, but interested readers may wish to consult several recent excellent reviews that specifically cover this topic [212-216]. Likewise, there are excellent reviews covering the entire spectrum of the pathophysiology of early and late menopause [217-221].

As noted before, the ovarian sex steroids, estrogens, androgens and progestin are primarily synthesized in the ovarian follicles. The follicle possesses two distinct steroidogenic cell types, which are highly compartmentalized but cooperate functionally to biosynthesize from cholesterol, progestin, androgens and estrogens in a sequential manner, with each serving substrate for the subsequent steroid production in the pathway [222,223]. The LH responsive theca cells are re-

sponsible for the production of androgens mainly androstenedione, which serves as a precursor for estrogen production in follicular granulosa cells by a process commonly referred to as aromatization [224]. These cells express relatively high-levels of three enzymes, cholesterol side-chain cleavage (P450scc, CYP11A), 3β-hydroxysteroid dehydrogenase (3βHSD) and 17α-hydroxylase/17,20 lypase (P450c17, CYP17) which sequentially catalyze the conversion of cholesterol to pregneolone to androstenedione [225]. The theca-derived androgens are transported to the adjacent granulosa cells for their conversion to estrogens.

The granulosa cell steroidogenesis and aromatization of androgens (androstenedione and testosterone) to estrogens is regulated by FSH during the follicular phase and LH during the luteal phase of the cycle. The granulosa cells) are also responsible for progesterone synthesis. These cells undergo hormone-mediated differentiation in the luteal phase of the menstrual cycle, transforming them from the primarily estrogen producing cells to mostly progesterone secreting cells. Likewise, granulosa cells exhibit high expression of aromatase activity during the follicular phase and high 3βHSD during the luteal phase. The granulosa cells do not express 17α-hydroxylase/17,20 lypase activity, thus necessitating the need for the development of an integrated process involving two cells (theca and granulosa cells) and two gonadotropins (LH and FSH) to facilitate estrogen production.

Changes in the circulating levels of ovarian steroids, estradiol and progesterone together with those in the levels of circulating of gonadotropins, LH and FSH during the normal menstrual cycle have been well documented [226]. In brief, plasma levels of estradiol begin to rise around the mid-point of the follicular or proliferative phase, increasing almost linearly for the last few days, and reach a maximum level 1 day before ovulation (i.e., LH and FSH peaks) [226,227]. Thereafter, estradiol levels decline rapidly, rise slightly during the middle of the luteal or secretory phase and, subsequently, decline rapidly reaching a basal level shortly before the initiation of menstruation and the next cycle [226,211]. The plasma levels of progesterone remain low in menstrual phase and follicular phase, but rise steadily following ovulation reaching maximum levels around the middle of the luteal phase and decline rapidly afterwards reaching a baseline shortly before the onset of the menses [226,227]. In contrast to these fairly predictable changes in estradiol, progesterone and gonadotropin levels throughout the menstrual cycle during the fertility period, such changes become highly erratic with variable relationships between sex steroids, LH and FSH, the occurrence of ovulation, and initiation of menstruation in response to the aging-induced menopausal transition (MT) and postmenopausal period [228-231].

The earliest evidence about the alterations in hormone levels occurring with advancing reproductive age was provided by Sherman and Korenman [232]. They reported that older women (46-51 years) undergoing MT (less functional ovarian follicles) had elevated levels of serum FSH along with decreased concentrations of estradiol, as compared to young women of 18-30 years of age [232,233]. LH and progesterone were comparable between the two groups [232,233]. Another study conducted by MacNaughton *et al* [234] reported that average FSH levels were two times higher in normal cycling women of 45-49 years of age as compared to three younger age groups. Serum estradiol levels were also lower in the same age group, but LH levels did not vary significantly in any of the age groups. Recent large-scale cohort studies conducted in Australia [235-238], Norway [239], and United States [240] have provided further important insights into the overall pattern of ovarian sex steroids and gonadotropins that occur during the MT. It is reported that circulating FSH levels rise progressively during the MT and that in perimenopausal women estradiol production fluctuates with FSH and often reaches higher concentrations than those in young normal cycling women below the age of 35. On the other hand, estradiol levels do not decrease appreciably until late in the MT. Despite the continuing regular cyclic menstruation, progesterone levels during the early MT are lower than in women of mid-reproductive age. Testosterone levels do not vary significantly during the MT.

By the time the postmenopausal period sets in, estrogen and progesterone decrease dramatically but testosterone levels decrease to a lesser extent [241,242]. The postmenopausal ovary still contributes approximately about 40-50% of all of the circulating testosterone [241-243]. Rannevik *et al* [244] carried out a longitudinal study in which 160 healthy 48-year-old enrolled women were followed for 12 years throughout the menopausal transition. They reported that both estradiol and estrone levels decreased significantly right at the onset of menopause. Following menopause, estradiol continued to show moderate but linear decline [242]. On the other hand, estrone levels did not change appreciably in response to menopause presumably due to increased peripheral aromatization of adrenal androstenedione, and which eventually became the primary source of estrogen during the postmenopausal years [242,244]. Significant decreases in the circulating levels of other sex steroids such as DHT, androstenedione and DHEA have also been reported in postmenopausal women as compared to women of reproductive age [243]. During the postmenopausal period, both LH and FSH concentrations remain highly elevated partially due to a decrease in negative feedback from ovarian steroids [228,229,245-247]. Elevated circulating levels of LH may also be responsible for enhanced androgen production by the theca cells as well as follicular atresia.

AGING AND GLUCOCORTICOID AND MINERALOCORTICOID SECRETION

Humans

Studies aimed at determining the impact of aging on basal, nonstimulated HPA function have produced mixed results. A majority of studies have shown that basal circulating levels of cortisol [248-266 and references therein] do not vary significantly with aging in humans. These analyses included the varying gender population as well as the health status of subjects with some using samples from males only [248,249,256,260-262], others employing both men and women [252-255,257,259,262,265], some from subjects classified as 'normal healthy' [248,249,256,260-262], and others from hospitalized patients [251,252,257,262]. Few studies have reported decreased [267,268], or increased [269-273] cortisol levels. Interestingly one report comparing the age-related changes in cortisol in men and women sug-

gests that women exhibit much greater age-related increases in cortisol secretion, with postmenopausal women showing the highest increases [269].

Numerous studies have reported that diurnal rhythmicity of cortisol [254,257,269-280] and ACTH [238,263] is also unaffected by aging in both sexes, but there are some exceptions. For example, some studies reported higher evening [251,281] and morning [278] cortisol levels at older ages; higher 24h mean cortisol concentrations at older ages have also been reported in both men and women [269,270,272, 281,282]. Other studies have reported no such age effect [280,283]. Interestingly, a recent study reported that 24-h cortisol levels were lower in men and higher in women with age [284]. This was attributed to decreased ACTH efficacy with age in men and increased ACTH efficacy with age in women [284]. Shifts in the timing of circadian peaks at older ages with later [285] or earlier morning peaks have also been reported [282,283]. Many follow-up studies particularly by Van Caulter *et al.,* [269], Dueschle *et al.,* [270], Yen and Laughlin [272], Kern *et al.,* [286], and Magri *et al.,* [287] further confirmed that circadian rhythms remain generally unchanged with age for ACTH and cortisol, but significant alterations do occur. The level of the nocturnal nadir cortisol progressively increases with aging [253,254,269,270], leading to a decline of the diurnal amplitude [269,270]. Moreover, the timing of the nocturnal nadir phase is advanced with aging [269,270,286,287], and the evening quiescent period shortened [269,270]. While the diurnal amplitude of ACTH also declines with age [270], ACTH levels were found to be either unchanged [287] or only moderately elevated [270].

Other studies suggest that aging augments ACTH and cortisol release in both sexes after selected stressors [288-292]. Raskind *et al.,* [293] compared serum cortisol levels in young and elderly individuals in response to an infusion of hypertonic saline. The older group had a higher cortisol level after the infusion leading to the conclusion that aging is associated with an increased cortisol release to stimulatory events. Gotthard *et al.,* [290] reported that cognitive challenges caused a greater rise in cortisol in the elderly than in the young. Consistent with this, Born *et al.,* [289] reported that basal levels of ACTH were lower in the younger subjects compared to older male and female subjects and that older subjects of both sexes had much greater response to the CRF (corticotrophin releasing factor) stimulation. Furthermore, Peskind *et al.,* [294] found that the administration of the pharmacological doses of physostigmine leads to more robust increases in ACTH and cortisol in the older subjects than in the young raising the possibility that the elderly have a more responsive HPA (hypothalamic-pituitary-adrenal) axis. In addition, there are a large number of other studies describing the age-related alterations in human HPA response to ACTH or DEX challenge. These studies are nicely summarized in a review article by Seeman and Robbins [262] and will not be discussed here.

Experimental Animals

Extensive literature exists about the influence of aging on plasma glucocorticoid and mineralocorticoid levels, particularly rodent plasma corticosterone levels under basal conditions and in response to treatment with various stressors or ACTH. Table **4** summarizes findings of some of these reports. It is apparent that both basal and stress-induced corticosterone levels differ markedly among various strains of rats and mice and also among the same strain when examined by different laboratories. Some of these variations may be due to various compensatory mechanisms operating in rodent models, increased or decreased disposal rates of the corticosterone or changes in binding proteins. But, when specifically challenged *in vitro*, isolated adrenocortical cells [295-302] of older male rats of several different strains show significantly less hormone response to maximal ACTH or cAMP stimulation than do cells from young or adult animals. Further studies from our own laboratory have shown that these changes in steroid hormone production and secretion are not a function of reduced ACTH receptors, cAMP production, cAMP phosphodiesterase, steroidogenic enzymes or lipoprotein-mediated cholesterol delivery and the major alteration in the respective cell types occurs distal to cAMP generation [297,298,303-305]. In contrast, several reports from one laboratory, however, suggests that the adrenocortical cells isolated from aged female rats in fact possess an enhanced capacity to secrete corticosterone in response to ACTH stimulation than cells from young or adult animals [306-310]. Finally, there is also evidence for age-related impaired aldosterone secretion by the isolated rat adrenal capsules and glomerulosa cells [311,312].

AGING AND ADRENAL ANDROGEN SECRETIONS

Humans

Dehydroepiandrosterone (DHEA) and its sulfated form, dehydroepiandrosterone-sulfate (DHEAS) represent the major androgens secreted by the human adrenal gland [313-315]. They are commonly referred to as the adrenal androgens. Although a small fraction of the circulating pool of DHEA is derived from gonads, the adrenal produces the bulk of circulating DHEA (S) [313-315]. It has been reported that plasma DHEAS levels in adult men and women are 100-500 times higher than those of testosterone and are 1000 to 10,000 times higher than those of estradiol [3,4], thus, providing a large reservoir for conversion into androgens and/or estrogens in peripheral intraendocrine tissues. Indeed, it is estimated that 30-50% of total androgen in men is synthesized in peripheral tissue, whereas in women, peripheral estrogen formation may be even greater [317]. Both DHA and DHEAS, however, are relatively less biologically active than other androgens.

The DHEA(S) shows a most striking age-related synthesis and secretion pattern that are unique to humans and non-human primates [313-322]. In humans, it occurs at three life stages as classified by Conley *et al* [319]: a) in utero from the fetal zone (FZ) cells of the developing adrenal cortex; b) during adolescence with the onset of adrenarche and the development of the zona reticularis (ZR); and c) in ever decreasing amounts from the ZR with advancing age. Before birth, DHEA(S) are produced in the fetal zone of the adrenal cortex early in gestation and production therein increases progressively through the second and third trimesters [3,6,9,11-13]. Immediately after birth, circulating DHEA(S)

Table 4. Effect of Age on Plasma Corticosterone Levels in Rodents

Brown Norway (BN) Rats

- van Eekelen *et al.,* [430] reported that total plasma cholesterol measured in blood samples collected between 0700-0900 did not differ between young (3-6-mo) and old (30-33-mo) rats. Likewise, corticosterone responses to novelty stress over a 4-hour period were comparable between young and old rats.
- In a follow-up work, van Eekelen *et al.,* [431] demonstrated that stress-induced peak circulating corticosterone levels were not different between two age groups, but *in vivo* sensitivity of adrenal glands to *in vivo* $ACTH_{1-24}$ challenge was reduced in aged (29-30-mo) compared to young (6-mo) adrenal glands. However, neither the magnitude of response to $ACTH_{1-24}$ nor total capacity of the adrenal cortex to synthesize and secrete corticosterone in response to $ACTH_{1-24}$ stimulation was impacted by aging.
- Gruenewald *et al.,* [432] using young (3-mo), middle-aged (13-mo) and aged (23-24-mo) male Brown Norway rats showed that corticosterone levels over a 24-hour period significantly and progressively declined with advancing age.

Fisher 344 (F344) Rats

- Sencar-Cupović and Milković [433] observed no changes in plasma corticosterone levels in response to advancing age.
- Landfield *et al.,* [434] observed significant increases in aldosterone and corticosterone levels in the mid-aged (13 mo) as compared to the young (4 mo) Fisher 344 rats. In the aged (25 mo) animals, the circulating levels of these corticosteroids were reduced to levels similar to those seen in young animals. However, the mean plasma concentration of corticosterone was elevated in aged rats.
- Brett *et al.,* [435] found no change with age (young 3- to 6-mo vs old 24- to 27-mo) in either sex in basal corticosterone levels, no change with age in males in stress-induced increments in corticosterone, and no change with age in males in stress-induced elevations in corticosterone, but a reduction in elevations induced by more potent stressors.
- Sapolsky *et al.,* [436] reported elevation of basal corticosterone levels with advancing age (ranging from 3- to 27 months of age). Older animals showed no impairment in their capacity to respond to stressors such as cage transfer, cold exposure, or immobilization stress. More importantly, the authors demonstrated that older animals recover from and adapt to stress in a delayed and incomplete manner.
- Sapolsky *et al.,* [437] noted comparable corticosterone levels in aged (24-26 mo) and young (3-5 mo) control rats under both basal and stressed conditions. In addition, it was reported that at the cessation of stress, corticosterone levels in young animals declined to basal levels within 90 min, whereas old animals still displayed high corticosterone levels even after 3h.
- Sapolsky *et al.,* [438] reported that aged rats (24-28 mo) secreted excessive amounts of the corticosterone under basal conditions as compared to young mature (3-5 mo), following the end of stress and during habituation to mild stressors. The old rats also showed resistance to inhibitory effects of the synthetic glucocorticoid dexamethasone upon subsequent corticosterone secretion.
- Sonntag *et al.,* [439] found no age-related changes in the diurnal rhythm or concentrations of plasma corticosterone, when plasma samples were analyzed from young (3-4 mo), middle-aged (10-12 mo) and old (22-24 mo) male rats.
- As reported by Lorens *et al.,* [440], the basal plasma corticosterone levels in the young (7 mo) and aged (22 mo) rats did not differ significantly when measured between 09:00-13:30. In contrast, the stress-induced increase in corticosterone levels were of greater magnitude in aged rats (155% compared to their age-matched control) as compared to young rats (88% higher compared to young control group), suggesting that stress leads to an exaggerated corticosterone in the aged rats.
- Irwin *et al.,* [441] reported that basal concentrations of plasma corticosterone and stress responses were similar in aged (24-mo) and young (4-mo) old F344 rats.
- Cizza *et al.,* [442] showed that basal corticosterone levels were lower in aged (24-mo) old rats as compared to young mature (8-mo) old rats. It was further shown that ACTH-induced corticosterone response was significantly lower in the middle-aged (18-mo) and aged (24-mo) rats when compared against young (2-mo) or young mature (8-mo) old rats.
- Hauger *et al.,* [443] reported that elevated diurnal trough levels of corticosterone in aged rats (24 mo) compared to young rats (4 mo). During the circadian peak in the evening, aged rats exhibited a small but significant reduction in circadian peak corticosterone compared to the young rats.
- Morano *et al.,* [444] provided evidence that young (5-6 months) and old (26-27 months) exhibited similar circadian rhythm of plasma corticosterone. Furthermore, they demonstrated that aged rats exhibit an aberrant stress response during both the light phase and the onset of the dark period of the cycle.
- Cizza *et al.,* [445] reported a significant decline in plasma levels of corticosterone in response to advancing age. Treatment (iv) with two doses of CRF (2 and 20 μg/kg BW) resulted in significantly greater corticosterone responses in older rats (18-mo and 24-mo). Treatment with $ACTH_{1-24}$ (0.5 mg/kg, iv) evoked a lower corticosterone response in older (18-mo and 24-mo old) that in the younger (2-mo and 8-mo old) groups of rats.
- Mastorakos *et al.,* [446] presented evidence that basal plasma corticosterone levels were similar in young (3-4 mo) and old (24 mo) female Fischer 344/N rats. Immobilization resulted in dramatic increases in corticosterone levels in both age groups. Furthermore, post-immobilization plasma corticosterone levels were found to be significantly higher in young as compared to old rats.
- Mulchahey *et al.,* [447] reported that basal corticosterone levels in aged (24 mo) rats were significantly elevated at both the AM (0700-0800 h) and PM (1800-1900 h) time points as compared to young (4 mo) rats.
- The work of Silverstein *et al.,* [448] demonstrated that stress significantly increased plasma corticosterone levels by 928%, 270% and 665% in 7-, 16- and 23-month-old rats, respectively. Furthermore, plasma corticosterone was increased by 174% in 23-month-old compared to 7-month-old young rats in response to stress. In addition, basal corticosterone levels were robustly increased in 16-month and 23-month old rats as compared to 7-month old rats.

Fisher 344/Brown-Norway F1 hybrid Rats (F344/BN F1 hybrid rats)

- The Taglialatela *et al.,* [449] reported significant increases in basal corticosterone levels in 18- and 30-mo old F344/BN F1 hybrid rats relative to young (3-mo) rats.
- Hebda-Bauer *et al.,* [450] utilized young (4-6-mo), old (23-25-mo) and very old (31-mo) F344/BN F1 hybrid rats to examine the effect of aging on circulating corticosterone levels. No age-related differences were noted in baseline, peak-time 2, and clearance of plasma cortiocsterone levels. Very old rats, however, showed significantly lower peak-time 1corticosterone levels than the young animals (4-6-mo).
- The authors employed young (3-mo), middle-aged (15-mo) and aged (30-mo) F344/Brown-Norway hybrid rats in their studies [451]. They determined the effect of age on plasma corticosterone levels in response to 3 stressors: restraint, spatial novelty and hypoxia. Compared to young (3-mo) and middle-aged (15-mo), aged-rats (30-mo) exhibited elevated corticosterone secretion in response to spatial novelty, but not in response to restraint or hypoxia. In contrast, middle-aged responded to chronic stress with increased corticosterone secretion in following hypoxia but not novelty.
- The Kascknow *et al.,* [452] examined corticosterone responses in 3-, 15-, and 30-mo old F344/BN F1 hybrid rats. Basal plasma corticosterone levels did not change with age. Similarly, corticosterone response to restraint stress was also unaffected by the advancing age. However, at 8 h after a dexamethasone challenge, corticosterone response in 30-mo old rats was significantly lower from that of the 3-mo old rats.
- Young (4-mo), middle-aged (12-mo) and aged (24-mo) F344 rats and aged (24-mo) and aged F344/BN F1 hybrids were used here [453]. Significantly higher plasma corticosterone levels were noted in aged (24-mo) F344 rats as compared to 4- and 12-mo old F344 rats. Likewise, corticosterone levels in 30-mo old F344/BN rats were significantly higher than cortiocsterone levels in 4-mo old F344 rats.

Table 4 Contd…..

Hooded Rats

- Spencer and McEwen [454] showed that there was no difference in basal plasma corticosterone levels between young (2-3-months) and aged male (22-23-months) hooded rats.

Long-Evans (LE) Rats

- Hess and Riegle [455] found no change in basal corticosterone levels in young (4-mo) female vs old (25-mo) female (Long-Evans) rats and young (6-mo) vs old (22-mo) male rats. However, both old female and male rats secreted significantly less corticosterone in response to either ACTH treatment or ether stress compared to respective young (control) animals.
- The work of Riegle and Hess [456] and Riegel [457] suggest that the ability of aged (22-32-mo) male and female rats (Long-Evans) to a negative feedback inhibition of the hypothalamic-pituitary axis is significantly impaired as compared to young (4-6-mo) adult animals.
- Meaney *et al.,* [458,459] reported elevated corticosterone levels in 16- and 24-mo old male Long-Evans rats as compared to young (3-mo) and adult (8-mo) rats. They also showed a tendency for increased hypothalamic-pituitary-adrenal activity (i.e., blunted feedback inhibition of HPA).
- Issa *et al.,* [460] demonstrated that basal corticosterone levels were significantly higher in cognitively impaired (AI) 23-27 month old male Long-Evans rats than those of cognitively unimpaired (AU) 23-27 month old rats or control (6-mo) animals. Evaluation of stress-induced changes in HPA activity showed that: a) basal pre-stress corticosterone levels were significantly higher in the AI animals; b) there were no group differences in the peak levels of corticosterone attained during stress; and c) AI animals had significantly higher plasma corticosterone levels at each point following the termination of the stressor.
- Rakotondrazafy and Brudieux [461] reported that plasma aldosterone incremental response to angiotensin II was significantly lower in aged (28-32-mo) female Long-Evans rats than in adult (8-10-mo) rats.
- Brudieux *et al.,* [462] examined age-related changes in plasma corticosterone and aldosterone responses to exogenous corticotrophin-releasing hormone (CRH) in female Long-Evans rats. Basal plasma corticosterone and aldosterone levels did not differ between young adult (7-9-mo) and aged (30-35-mo) rats. Treatment with CRH markedly increased plasma levels of corticosterone and aldosterone with similar kinetics, but incremental responses at each time points in old as compared to adult rats.
- Ait-Chaoui *et al.,* [463] observed no age-related changes in basal corticosterone and aldosterone levels in both male and female Long-Evans rats. Furthermore, the authors reported a blunting of corticosteroid (corticosterone and aldosterone) to ACTH in aged male/female (25-31-mo) rats as compared to young male (3-4-mo)/female (4-5-mo) or adult female (12-14-mo) rats.
- Bizon *et al.,* [464] reported finding no differences in either basal or peak plasma corticosterone levels were observed between young (6-mo) or aged (27-28-mo) male Long-Evans rats.

Sprgue-Dawley (SD) Rats

- Lewis and Wexler [465] observed elevated corticosterone levels in middle-aged (15-18-mo) rats versus young (3-4-mo) rats.
- Britton *et al.,* [466] reported a 75% decline in circulating corticosterone levels as rats aged from 2-mo to 24-mo.
- Tang and Phillips [467] found no age-related differences in either basal or stress-stimulated corticosterone levels.
- DeKosky *et al.,* [468] measured serum corticosterone levels at three time points in the diurnal cycle (08:00, 18:00, and 23:30 h) using young adults (3-5-mo), middle-aged (14-16-mo), and aged (26-28-mo) rats. In aged animals, serum corticosterone levels at both 18:00 h and 23:30 h were elevated as compared to young rats with most dramatic aging noted at 23:30 h. Moreover, these high levels persisted for a longer time.
- Scaccianoce *et al.,* [469] observed higher circulating levels of corticosterone in aged (25-mo) relative to young (3-mo) rats.
- Foreman *et al.,* [470] provided data showing elevated corticosterone levels in aged (23-mo) compared to young (3-mo) rats. In addition, it was reported that the magnitude of the stress-stimulated corticosterone levels were significantly higher than of similarly treated young animals.
- Terrazino *et al.,* [471] reported higher basal corticosterone levels in old (24-mo) as compared to young (3-mo) rats ($p<0.01$). In contrast, decreased corticosterone secretion in old rats was noted in response to LPS stimulation.
- Studies by Núñez *et al.,* [472] revealed elevated basal corticosterone levels in old female (19-22-mo) compared to young (4-6-mo) female rats. However, isolation stress-induced increases in corticosterone levels were similar between young and old rats. In contrast, stress-stimulated corticosterone levels declined more rapidly in young than aged rats following the cessation of isolation stress.
- Lo *et al.,* [473] demonstrated that plasma corticosterone levels were significantly increased in middle-aged (12-mo) and aged (22-24-mo) as compared to young (3-mo) or young mature (6-mo) rats. However, no age-related differences in ACTH stimulation of plasma corticosterone levels were noted among the four groups. Interestingly, two hours after ACTH treatment, plasma corticosterone levels were relatively elevated in old rats compared to young mature rats. Montaron *et al.,* [474] reported an age-related increase in basal plasma corticosterone levels [i.e., young (3-mo) or young mature (14-mo) vs old (21-26-mo). However, no aging effect on plasma corticosterone levels was detected when animals were subjected to restraint stress.

Wistar Rats

- Korte *et al.,* [475] reported that basal plasma corticosterone levels were slightly higher in the aged rats (24 mo) compared to young (3-mo) controls, but the differences were not statistically significant. Both young and old rats showed similar increases in plasma corticosterone levels in response to a single foot shock. Interestingly, one day after shock, the plasma corticosterone levels were reported to be significantly higher than that of controls. On the other hand, no significant differences were noted in the plasma corticosterone levels of young and old rats when challenged with the conditioned defensive burying (CDB) test.
- Yau *et al.,* [476] provided evidence that the basal plasma corticosterone levels were unaffected in aged rats (18-24 mo) as compared to young (7-months) rats.
- Mizuno *et al.,* [477] reported that in aged rats (23-24 mo), basal concentrations of corticosterone were significantly elevated than that in the young (3-4 mo) rats. In young rats, but not in old rats, plasma corticosterone levels were increased in response to stress.
- Bazhanova *et al.,* [478] reported that young (3-6 mo) and old (28-mo) rats had similar levels of basal corticosterone levels. Furthermore, it was noted that stress-induced corticosterone levels also did not change with age.
- Descamps and Cespuglio [479] showed that immobilization stress led to greatly increased plasma corticosterone levels in both young (3-mo) and old (24-mo) rats, although aging had no effect on such responses.
- Garrido *et al.,* [480] provided evidence that the basal levels of total plasma corticosterone measured in blood samples were not different between young (6-mo) and aged (27-mo) rats. Restraint stress significantly increased plasma corticosterone levels in both young and old rats, although the average increase in corticosterone levels in response to stress was significantly higher in old rats.

BALB/c Mice

- Buchanan *et al.,* [481] examined the effect of repeated stress on plasma corticosterone levels in adult (3-5 months) and aged (22-24 months) male BALB/c mice. It was reported that aged-mice had higher levels of basal plasma corticosterone compared to adult mice. They also showed that in adult mice, plasma corticosterone increased to a greater extent with each successive stress session, whereas in aged mice, two stress sessions produced the maximum increase in corticosterone.

Table 4 Contd…..

C57BL/6 Mice
• Padgett *et al.,* [482] showed that restraint stress caused conparable increases in circulating plasma corticosterone levels in control (3-mo) and aged (22-mo) male C57BL/6 mice, but old mice required a longer time to attain baseline plasma corticosterone levels following the removal of a stressor.
• Rosenthal and Morley [483] provided data showing that aging has no significant effect on plasma corticosterone levels, i.e., plasma corticosterone levels were similar in adult (10-mo) and aged (27-mo) male C57Bl/6 Nnia mice both under basal condition and in response to CRF stimulation.
• Studies by Waziers *et al.,* [484] demonstrated that plasma corticosterone levels were significantly higher in young control (2-mo) as compared to aged.
• Park *et al.,* [485], reported no age-related alteration in plasma corticosterone levels in female C57BL/6J mice both under basal condition and in response to extended restraint stress. For these studies mature adult control (7.5-mo) and aged (22-mo) C57BL/6J female mice were employed.
• Dalm *et al.,* [486] employed 3-, 9-, and 16-month old C57BL/6J mice to examine the age-related changes in hypothalamic-pituitary-adrenal (HPA) activity. The highest AUC values over 24h periods were shown by 9-months old mice as compared to 3-months and 16-months old mice. The three age groups showed a distinct circadian pattern; during the light period, 9-month old mice secreted highest levels of corticosterone followed by 3- and 16-months old mice. The corticosterone secretion rates during the dark phase were not impacted by aging.
C3B10RF$_1$ Hybrid Strain of Mice
• Harris *et al.,* [487] found no age-related changes in plasma corticosterone levels in the long-lived C3B10RF$_1$ hybrid strain mice. These studies employed 7-, 17- and 29-month old female mice.
CF-1 Male Mice
• Thurmond and Heishman [488] assessed the secreted corticosterone levels in young (3-mo) and aged (30-mo) CF-1 male mice after cold swim stress. They showed that circulating levels of corticosterone increased to a greater extent in young than aged CF-1 mice.

levels are high due to their synthesis by the fetal adrenal gland. However, DHEA(S) levels drop rapidly as a result of fetal zone atrophy and its disappearance in the first year of life [320,321,323-325]. During childhood, DHEA(S) concentrations remain low through early childhood, but by about 6-7 years of age (with the development of ZR cells in the adrenal cortex), the plasma DHEA(S) concentrations begin to rise and about 2 years before the onset of puberty, there is a dramatic increase in DHEA(S) secretion, termed the 'adrenarche' [316,317,321]. The plasma levels of DHEA(S) peak at approximately 20 years of age and decline progressively after the age of 25 [318] to 5-10% of young adult (peak) levels peak values [3,6] by age 70-80. In general, the age-related decline in DHEA(S) levels shows high inter-individual variability and seems to be associated with a loss of zona reticularis cells and/or impairment of their function [318,325]. This phenomenon is referred to as adrenopause, and it occurs in both men and women with a gradual pace at similar ages.

Non-human Primates

Besides humans, the fetal adrenal gland and adult zona reticularis (ZR) of non-human primates also secrete mg quantities of DHEA(S) [319,321,326]. Although various non-human primates show some degree of age-associated secretion pattern in DHEA(S), until now none of the non-human primates have been identified that faithfully duplicate the human adrenal secretion pattern covering all three life stages (i.e., fetal zone or FZ, adrenarche, and senescence) [319,321,326]. However, based on the limited morphological, biochemical, and endocrine data, however, it appears that the marmoset simulates the human pattern of FZ, the chimpanzee the human adrenarche, and the Rhesus macaque or Rhesus monkey and baboons the human mature ZR function that progressively declines with senescence [319,321, 326].

Rodents and Other Mammals

In contrast to humans and non-human primates, rodent adrenals lack P450c17, and hence cannot synthesize DHEA [327]. Because of the P450c17 deficiency, circulating levels of DHEA and its sulfated form, DHEAS, are several orders of magnitude lower than in humans. Furthermore, no age-related changes in plasma DHEA levels have been documented in rodents [328]. Likewise, studies by Cutler *et al* suggest that circulating concentrations of DHEA(S) remain low throughout life in most mammals including the guinea pig, hamster, rabbit, dog, sheep, pig, goat, horse, and cow [329].

OXIDATIVE STRESS AND AGE-RELATED DECLINE IN STEROIDOGENESIS

The extensive evidence as presented above indicates that aging in humans and experimental animal models is associated with a general decline in steroid hormone production. Work over the years from this laboratory has shown that the basic problem, at least in aging rats, is that adequate amounts of cholesterol are not available to adrenal (adrenocortical cells) and testis (Leydig cells) for the first step in steroid biosynthesis (i.e., conversion of cholesterol to pregnenolone by the inner mitochondrial membrane-associated side-chain cleavage enzyme system, P450$_{scc}$ [also designated as CYP11A1]) [25,53,58,66,76,90]. Questions now center on the nature of this defect. It appears that the uptake of lipoprotein-derived cholesterol esters in the adrenal of aging rats is entirely normal and ample [304,305], and that overall, the accumulated stores of steroid precursor, cholesterol in both adrenocortical cells and Leydig cells from aging animals is more than adequate [183,298]—yet the mitochondria of the steroidogenic cells of these animals do not receive adequate cholesterol substrate.

A substantial body of evidence has accumulated over the last few years indicating that levels of both StAR and PBR/TSPO (the two important intracellular molecules that assist in mediating the cholesterol transport process) are decreased in aging rats—linking an alteration in sterol transfer and transport proteins with the age-related defect in steroidogenesis. When these issues were explored further, it became evident that increased ROS production (primarily from impaired anti-oxidant defense systems) leads to changes in expression of sterol transfer proteins (StAR, StAR-related proteins [StarDs] and PBR/TSPO] in steroidogenic tissues of

aged animals, and downstream, this leads to a reduction in cholesterol transfer to mitochondria sites where cholesterol side chain cleavage takes place. In the following sections, we summarize the impact of aging-induced excessive ROS generation and ensuing excessive oxidative stress on the events connected with the cholesterol transport to mitochondria for steroid hormone production.

Theories of Aging

Physiologically aging is an extremely complex, multifactorial process, affecting a myriad of genetic, biochemical, and metabolic processes. Although, numerous aging theories have been proposed to explain mechanisms of aging [330], no theory has been more lasting in this regard than that known as the "free radical theory of aging," in which damage by free radicals (reactive oxygen species [ROS]) is deemed critical in determination of life span [331-333]. This theory proposed in 1956 by the Denham Harman [334] states that free radicals, especially ROS formed as by-products of normal metabolic processes, cause oxidative damage to macromolecules, whose accumulation causes cellular dysfunction, and this ultimately determines the lifespan of an organism [335,336].

Over time, the free radical theory has been further refined; first it was modified to become the "oxidative stress theory" of aging to reflect the fact that oxygen species such as peroxides and aldehydes, which do not fall under the category of free radicals, also play a role in oxidative damage to cells [337]. Later with the realization that mitochondria are at the same time major sources and targets of ROS, the theory was renamed as the "mitochondrial theory of aging" [331,332,335,337,339]. According to this theory of aging, ROS produced as by-products of the mitochondrial oxidative respiratory chain damage mitochondrial macromolecules, especially mitochondrial DNA (mtDNA). As a result, an accumulation of mtDNA mutations leads to production of defective mitochondrial respiration, further increasing ROS generation and oxidative damage. It is also suggested that accumulation of oxidant-induced somatic mutations alter the mitochondrial respiratory complex function leading to increased ROS production and further damage to mtDNA as well as other macromolecules [340,341]. This "vicious cycle" of ROS generation and concomitant oxidative damage to macromolecules is suggested as the principal determinant of mammalian lifespan. Over the past few decades a significant amount of correlative evidence has been accumulated in support of the mitochondrial theory of aging including studies that show an increase in ROS generation with age, the age-related increase in oxidative damage to DNA including mtDNA, lipids, and proteins, increased mtDNA deletions and mitochondrial dysfunction with age [338,340].

In recent years, however, the use of transgenic and knockout mouse models with altered expression of antioxidant enzymes and mutant mouse models that have been genetically modified to increase mitochondrial deletions or mutations ($Poly^{D257A/D257A}$ mutant mice) to directly test the validity of the mitochondrial theory of aging have yielded inconsistence results [337,342-346]. Moreover, in some instances, the data obtained failed to directly support a direct role for mitochondrial oxidative stress or oxidative stress in the determination of life span in mice and also did not support the overall free radical theory of aging [337,342-346]. Although, these various findings are somewhat disappointing, more recent mechanistic studies, however, have clearly implicated the mitochondrial free radical damage and impaired mitochondrial function as being a central contributor to the pathophysiology of aging [347-349]. One prevailing view is that mitochondria influence longevity not only through increased production of ROS and ensuing oxidative damage but also *via* a stress (ROS)-evoked signal that acts in a cell-non-autonomous manner to regulate mitochondrial protein homeostasis [347,348]. The other studies emphasize a link between excessive ROS production and the functional efficacy of insulin signaling, which are based on the epigenetic oxidative redox shift (EORS) theory of aging [349]. According to EORS, which unifies the free radical and insulin signaling theories, sedentary behavior associated with age triggers an oxidizing redox shift, enhanced ROS generation, reduced mitochondrial turnover and impaired mitochondrial function [349], culminating in further accelerated aging.

Aging and Excessive Oxidative Stress

Based on extensive *in vivo* and *in vitro* studies, it now appears that senescence is associated with increased oxidant generation, a decline in the robustness of cellular defenses and repair, and an accumulation of the end products of the oxidative damage [331,336,339-354]. In general, the three main classes of biological macromolecules (lipids, nucleic acids and proteins) are susceptible to free radical attack and suffer oxidative damage *in vivo* [331,336,339-354]. Because cellular membranes house the production of these radicals, membrane lipid peroxidation is now regarded as the major process that produces ROS damage during aging [354,355]. This idea is reinforced by the fact that lipid peroxidation increases with age [331,336] and that oxidized lipid residues are major components of lipofucsin, the fluorescent pigment that accumulates with age in most tissues [331].

The risk of lipid peroxidation is especially high for steroidogenic cells, which use molecular oxygen for steroid biosynthesis [356,357] in addition to a more standard cell function [353-355,358]. Cytochrome P450 enzymes of the steroidogenic pathway use molecular oxygen to hydroxylate substrates. As this occurs, ROS such as superoxide anions, hydrogen peroxide, hydroxyradicals and other oxygen free radicals can also be formed as a result of electron leakage or by interaction of the P450 hydroxylases with steroid products (or pseudosubstrates) [356-361]. These oxy-radicals combined with a high content of target substrates for ROS (such as polyunsaturated fatty acids in the adrenal gland and gonads) exaggerate the potential of oxidative changes leading to cell damage and death. Because lipid peroxidation involving membranes could affect membrane composition, structure, fluidity and function (e.g. the activation of membrane proteins like receptors, ion channels, participants of signal transduction pathways, transport proteins and membrane associated enzymes [362-366]), and virtually every event associated with cholesterol processing and steroidogenesis is dependent on the integrity of cell membranes [1-6,25,26,53,81,90,367-369], the likelihood of steroidogenesis being adversely affected is quite high. As a result, steroidogenic cells, like other mammalian cells, are well equipped

with antioxidant systems to combat free radicals [370-375]. These antioxidant systems are comprised of 1) low molecular weight agents such as vitamin E (atocopherol), vitamin C (ascorbic acid), reduced glutathione (GSH), carotenoids, flavonoids, uric acid, bilirubin, and lipoic acid; 2) iron and copper sequestering proteins such as transferrin, lactoferrin, hemopexin, albumin, and ceruloplasmin; 3) antioxidant enzymes such as superoxide dismutase (Cu,Zn-SOD, Mn-SOD), glutathione peroxidases (e.g., cytosolic glutathione peroxidase [cGPX], phospholipid hydroperoxidase glutathione peroxidase [PHGPX], plasma glutathione peroxidase [pGPX]), catalase, heme oxygenase, and thioredoxin reductase (TR); and 4) accessory antioxidant proteins such as thioredoxins, glutaredoxins, and peroxyredoxins [331,350-353,376-381].

Oxidative Damage and Oxidant-sensitive Transcription Factors

Indeed, this laboratory has shown that adrenals from young rats show the least endogenous lipid peroxidation and the highest levels of resistance to pro-oxidant-induced oxidative damage of the various tissues examined, and show exceedingly high levels of tissue antioxidants [372]. Despite this, aging leads to many oxidative changes both in the adrenocortical and the testicular Leydig cells [372,374]. These are linked, with time, to a dramatic reduction in the normally protective antioxidant defense system, thus, leading to excessive oxidative stress, and, we believe, ultimately to the decline of steroid production in the aging animals, i.e., the increase in lipid peroxidation in steroidogenic tissues may be the underlying cause of the age-related decrease in corticosterone/testosterone synthesis. It is unclear why steroidogenic cells do not respond to this oxidant balance by simply modulating the expression of antioxidant enzymes [372,374], oxidant-sensitive transcription factors, AP-1 and NF-κB [353,358,382-385] and other stress inducible cytoprotective genes invoking cellular protective mechanisms [386]. We speculate that up-regulation does not occur in the aging animals because the continuous oxidant burden likely overwhelms the cells, leading to the failure of cellular transcriptional/translational machinery and, thus, the function of various proteins involved in the transport of cholesterol to the mitochondrial sites of P450scc. Experimental evidence that this is the case comes from earlier reports from our laboratory showing that the expression of oxidant-sensitive transcription factors, the activator protein-1 (AP-1) and nuclear factor κB (NF-κB) are both substantially reduced in adrenal extracts from aging rats [387,388].

AP-1 Transcription Factor

The transcription factor AP-1 mediates gene regulation in response to a broad range of physiological and pathological stimuli, including cytokines, growth factors, ROS, infection and other stress signals as well as oncogenic stimuli [353,385,389-394]. In addition, steroid producing tissues are thought to require AP-1 for the regulation of steroidogenesis [387,385-400]. AP-1 transcription factors are homo- and hetero-dimers of Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, FosB splice variants FosB2 and DeltaFosB2 and Fra-1 and Fra-2) Jun dimerization partner (JDP1 and JDP2) and the closely related activating transcription factor (ATFa, ATF2, LRF1/ATF3, B-ATF) family of proteins characterized by basic region and leucine-zipper domains [353,385,389-394]. In addition, some of the Maf proteins (v-Maf, c-Maf and Nrl) can heterodimerize with c-Junior c-Fos, whereas other Maf related proteins, including MafB, MafF, MafG and MafK, heterodimerize with c-Fos but not with c-Jun. While Jun proteins can themselves form stable homodimers, Fos family members are not able to form homodimers, but homodimerize with Jun partners, giving rise to various *trans*-activating or *trans*-repressing complexes with different biochemical properties. Together, all these proteins form the group of AP-1 proteins that after dimerization bind to the AP-1 DNA recognition elements (5'-TGA$^{G}/_{C}$TCA-3'), also known as 12-*O*-tetradecanoylphorbol-13 (TPA)-response element (TRE) in the promoter and enhancer regions of the target genes [389-391,393,394].

Members of the ATF family of proteins can form homodimers or heterodimers with AP-1 proteins (predominantly with Jun proteins) that preferentially bind to cAMP-responsive elements (CRE, 5'-TGACGTCA-3'). AP-1 activity is regulated at various levels, including transcriptional and post-transcriptional mechanisms leading to increased AP-1 expression and post-translational modifications, such as phosphorylation, post-translational processing and turnover or pre-existing or newly synthesized AP-1 subunits and oxidation/reduction, altering DNA binding affinity and transactivation potential [390,392-394,401-405]. Since DNA binding affinity and transactivation potential are different for the various proteins, AP-1 activity is also determined by its composition [390,393,403,405]. Thus, the extent of transcriptional activation or repression conferred upon AP-1 responsive genes depends on post-translational modifications, selective dimerization between different family members, and protein-protein interactions with other regulatory molecules. AP-1 function is also cell type specific [390,393,403,405].

Our own studies demonstrate that the expression of AP-1 constituent proteins (Fos and Jun family of proteins) are considerably reduced in the adrenal extracts of 24-month old rats , and that adrenal expression of JunB (normally considered a repressor of transcription, a cell proliferation inhibitor, a senescence inducer and tumor suppressor [406-410]), is greatly increased [387]. All the AP-1 Fos/Jun family of proteins in adrenal extracts are dramatically activated by lipopolysaccharide (LPS) (a pro-oxidant stressor), as anticipated for agents regulating oxidative stress events, but significantly activation occurs in adrenal extracts of aging (24-mo-old rats) compared to that of 5-mo-old mature rats [387].

Aging and p38 Subfamily of MAP Kinases

The p38 subfamily of MAP kinases consists of 4 members, p38α, p38β, p38γ and p38δ MAPKs, which share high sequence homology and a signature TGY, where T, G and Y are threonine, glycine and tyrosine, respectively [412-415]. Phosphorylation of both the threonine and tyrosine within this signature sequence is required for p38 MAPK activation. Phosphorylation of p38 MAPKs *via* a signaling cascade involving MAPK kinases [MKK3/MKK6] is responsible for phosphorylation of p38 MAPKα,β,γ, and δ, and MAPK

kinase kinases [MAPKKKs] that phosphorylate and activate MKK3/MKK6. The activated [phosphorylated] form of p38 MAPK phosphorylates a large number of transcription factors including the NFAT, p53, MEF2, and AP-1 family of proteins such as ATF2, c-Jun, JunB, and c-Fos).

Our recent Western blot analyses data indicated that the levels of active (phosphorylated) form of the p38 MAP kinase (p38 MAPK), in adrenal [411] and Leydig cell lysates (unpublished observation) were substantially increased with aging. The follow-up studies demostrtaed that exposure of Y1-BS1 mouse adrenocortical and the MLTC-1 mouse to various oxidative stressors preferentially stimulated p38 MAPK activity, but at the same time inhibited steroid production [416] and caused down regulation of AP-1 activity (unpublished observation). In addition, recently completed studies with the use of active (wild-type) and dominant negative (dn) cDNA constructs of the p38 MAPK signaling pathways (e.g. p38 MAPKα and MKK6) suggest that p38 MAPK is a negative regulator of the functional expression of AP-1 in steroid producing model cell lines.

Thus, it appears that aging in the rat adrenal [372] and testicular Leydig cells [374] is linked to a reduction in both enzymatic and non-enzymatic components of a cellular natural defense system against oxidative damage, as well as loss in the expression of major AP-1 transcription factor. Under normal stress conditions the expression of major AP-1 constituent proteins [382,402] is up-regulated, but in the aging adrenal, the only transcription factor whose expression is increased (rather than decreased) is Jun B [387], a putative repressor of AP-1 function [406-410]. These events suggest that cellular defense against oxidative stress is reduced in aging animals, and the potential impact of this on steroidogenesis becomes even more clear when one realizes that promoter regions of StarD1/StAR and PBR/TSPO genes, the two intracellular molecules which assist in mediating the cholesterol transport process [53,58,59,66,76,81,417-419], contain an AP-1 response element [420,421], and that the proximal AP-1 site in the StarD1/StAR promoter plays a pivotal role in regulating StarD1/StAR gene transcription [420].

It is well known that the acute, rate-limiting step in steroid hormone biosynthesis in steroidogenic tissues is the translocation of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where it is converted to the pregnenolone by the cholesterol-side chain cleavage enzyme, P450scc (CYP11A1), thus initiating the synthesis of the steroid hormone [1-6]. It can be demonstrated that when hydroxyl analogs of cholesterol [such as 22(R)-, 20α- or 25-hydroxycholesterol, which readily diffuse though cell membranes to the P450scc] are provided to cells, high levels of steroid can be produced even in the absence of hormone stimulation, i.e., it is not the reaction catalyzed by P450scc that is rate limiting, but rather the transport of cholesterol to these sites. The same is true for cells from aging animals. When supplied with freely diffusible cholesterol analog (i.e., hydroxyl cholesterols), adrenocortical and testicular Leydig cells from aging rats are totally competent in producing steroids (corticosterone and testosterone). The "acute" production of steroids is dependent upon a hormone-stimulated, rapidly synthesized, cyclohexamide-sensitive and highly labile protein whose function is to transfer cholesterol from outer to inner mitochondrial membranes [53,61-63]. StarD1/StAR is now identified as a putative acute regulatory protein [53,58,59,76,90,417,418]. Recent evidence, however, indicates that StarD1/StAR may also requires participation of a PBR/TSPO multimeric complex in facilitating cholesterol transport from the outer to the inner mitochondrial membrane [75,90,419].

In regard to the apparent critical function of an "acute" regulatory protein in steroidogenesis, it is of interest that expression of both StarD1/StAR and PBR/TSPO is down-regulated in the adrenal and Leydig cells of aging rats [422-426]. For example, studies from this laboratory have shown an 80% reduction in StarD1/StAR protein expression of non-stimulated Leydig cells prepared from old Sprague-Dawley rats suggesting that the impaired testosterone production that occurs during aging may involve attenuated StAR expression [422]. Upon stimulation with hCG, Leydig cells from young rats demonstrated a 3-4-fold increase in StAR expression (both protein and mRNA levels), while expression in cells from aging (24-mo-old) rats increased no more that 50-70%. Likewise, expression of StarD2 and StarD4 is reduced in aging Leydig cells, both under basal and hCG-stimulated conditions [426]. The expression of neither StarD3 nor StarD5 was affected by aging. Also, experiments using adrenals from aging animals show ACTH stimulated StarD1/StAR and PBR/TSPO mRNA expression is dramatically reduced (unpublished observation). At the same time, expression of DBI did not change, nor did the expression of the other putative sterol transfer factors such as SCP2, SAP precursor (GRP-78), VADC1-3 or ANC. The concept that insufficient sterol transfer protein in steroidogenic cells of aging animals explains the age-related impairment in hormone production is strengthened by studies of mutations in humans and the ablation of the StAR gene in mice. Similar to what is observed in aging adrenal and Leydig cells, humans carrying mutations that inactivate StarD1/StAR exhibit markedly reduced gonadal and adrenal steroidogenesis, a condition that leads to excessive accumulation of cholesterol in lipid droplets and a disease named congenital lipoid adrenal hyperplasia [427]. Ablation of the StarD1/StAR gene in mice also results in a phenotype of impaired steroidogenesis and adrenal lipid accumulation [428]. Likewise, deletion of PBR/TSPO results in a greater than 90% reduction in steroid production and severe impairment in cholesterol transport to mitochondria, both of which can be rescued by re-introduction of the PBR/TSPO [429].

It appears, therefore, that several critical inter-relating factors relevant to steroidogenesis are significantly altered in the aging rat model. These may include expression levels of sterol transfer proteins (such as StarD1/StAR, StAR-related proteins and PBR/TSPO), oxidant sensitive transcription factors (such as AP-1 possibly involved in the inducible expression of sterol transfer protein genes) and other genes responsive to oxidative stress. A significant attenuation in AP-1 expression over time and continual excessive oxidative insult might lead to impaired regulation of StAR gene transcription, decreased expression of other StAR-related cholesterol transfer proteins, and the StAR partner, PBR, and as a result, decreased cholesterol delivery to mitochondria and

loss of steroidogenesis. It remains to be determined as to how increased ROS formation and ensuing oxidative damage leads to changes in expression of sterol transfer proteins, StAR, StarD proteins and PBR/TSPO in steroidogenic tissues of aged animals, and downstream, this leads to the transfer of less cholesterol to mitochondrial sites where cholesterol side chain cleavage takes place.

CONCLUSION AND PERSPECTIVES

All steroid hormones are derived from cholesterol, and they are made up of three six-carbon rings, one five carbon ring, and a unique side chain, a basic structure that is characteristic of the cholesterol molecule. They are secreted primarily by three "steroidogenic glands"—the adrenal gland, ovaries and testes and during pregnancy by the placenta. Steroid hormones are classified into five classes namely: estrogens, progestins, androgens, glucocorticoids and mineralocorticoids. Glucocorticoids and mineralocorticoids together are called corticoids, whereas estrogens, progestin and androgens are often referred to as sex steroids. The androgens and estrogens are primarily responsible for the development and maintenance of reproductive function and secondary sex characteristics in the male and female, respectively. Estrogens and progestin are typically linked with the female reproductive functions such as menstrual cycle, ovulation and implantation of the embryo. Mineralocorticoids such as aldosterone help maintain the balance between water and salts in the body, predominantly exerting their effects within the kidney. Glucocorticoids are typically involved in the regulation of lipid, carbohydrate and protein metabolism.

Numerous cross-sectional longitudinal studies conducted during the past four decades or so have established that circulating levels of testosterone decline with advancing age in men, and this decline has been associated with parallel age-related metabolic and pathophysiological changes such as increased fat mass, decreased muscle and bone mass, frailty, depression, sexual function, osteopenia, and osteoporosis, insulin resistance, diabetes and increased cardiovascular risk. Although there is dysfunction of the hypothalamic-pituitary-gonadal (HPG) axis in men, the age-related decline in testosterone levels is most likely to involve direct alterations of testicular Leydig cell function. Likewise, aging of the human ovary also results in a gradual decline in ovarian steroid production, followed by an abrupt and complete cessation of both progesterone and estrogen production at the onset of menopause. Human aging is also associated with alterations in adrenal steroid secretion, a decline in aldosterone secretion and subtle changes in cortisol production. Also, the adrenal androgens, DHEA and DHEAS decline more dramatically with age in a situation similar to menopause, and these decreases are considered to aggravate some age-related diseases.

Similar to humans, advancing age in experimental animals, particularly rats, is also associated with profound changes in the synthesis and secretion of steroid hormones. With corticosterone or testosterone, one does not always notice a decline in circulating levels due to various compensatory mechanisms of the organism, decreased disposal rates of the hormones, or change in binding proteins, etc? But when specifically challenged *in vitro*, isolated adrenocortical cells or Leydig cells of older rats in several different strains show significantly less steroidogenic response to trophic hormone stimulation than do cells from young animals. Overall, these changes appear not to be a function of reduced tropic hormone receptor signaling or a defect in the steroid synthesizing, but a change in the ability of aging tissues to mobilize the intracellular cholesterol needed for steroid hormone production. Despite the fact that these accumulate excessive amounts of cholesteryl esters in the form of lipid droplets, it appears they cannot appropriately transport the steroid precursor (i.e., free cholesterol) to mitochondrial sites of the CYP11A1/P450scc enzyme, where the conversion of cholesterol to pregnenolone (the precursor of all steroid hormones) takes place with side chain cleavage. Although various cellular and molecular mechanisms controlling this aging defect have not been definitively identified, considerable evidence from this laboratory points to excessive free radical (ROS) formation and oxidative damage (especially from life-long continued processing of cholesterol for steroid production) to the cell machinery regulating the functional expression of crucial proteins involved in cholesterol transport. The latter include cellular sterol transport proteins (such as StarD1/StAR protein, certain StAR-related [StarD] proteins, and a multimeric protein complex collectively known as the PBR/TSPO complex, which assists in translocation of cholesterol from the OMM to the IMM, where it is converted to pregnenolone by mitochondrial side chain cleavage (CYP11A1/P450scc), as well as interactions between tissue AP-1 transcription factors and p38 MAPK, which appear to regulate the expression of the StarD1StAR protein, and possibly that of PBR/TSPO.

However, very little is known about the underlying molecular mechanisms by which aging-induced excessive oxidative stress alters the functional expression of these critical inter-relating factors relevant to steroidogenesis. Outstanding questions that should be addressed in the future include: (1) a detailed characterization of molecular events that lead to excessive ROS production and oxidative damage; (2) mechanism(s) by which aging-related continual excessive oxidative insults leads to a significant attenuation of AP-1 expression, and consequently, impaired regulation of StarD1/StAR and its partner PBR/TSPO and the relevant StarD proteins; (3) the involvement; and (4) the pathological and physiological roles of p38 MAPK isoforms in mediating age-related excessive oxidative stress-induced alterations in the functional expression of these crucial steroidogenic protein factors. The application of the state-of-the-art molecular, cellular and biochemical approaches, coupled with high-throughput proteomic and lipidomics approaches, may provide valuable insights into these important questions. Moreover, the use of genetically modified mice to modulate (decreased or increased) expression of antioxidant enzymes (e.g., Cu,Zn-SOD, Mn-SOD, catalase, GPX-1 or GPX-4) in a time-dependent manner will be very useful to elucidate *in vivo* mechanism(s) by which excessive oxidative stress contributes to age-related decline in steroid hormone synthesis and secretion. The use of designer cell lines overexpressing a pair of various combinations of antioxidant enzymes (e.g.

Mn-SOD/catalase or Mn-SOD/GPX-1 will convert superoxide anions into harmless water) or chronic treatment of old rats with small molecular weight natural or synthetic antioxidants should also yield valuable information. It is likely that future studies along these lines may identify novel targets for the development of new therapies to treat male hypogonadism and other clinical conditions associated with alterations in the level of specific steroid hormones in response to aging.

ABBREVIATIONS

ACTH	=	Adrenocorticotropic hormone
ANC	=	30-kDa adenine nucleotide translocator
AP-1	=	Activator protein-1
CEH	=	Cholesteryl ester hydroxylase
C	=	Adenylate cyclase
CEs	=	Cholesteryl esters
DEX	=	Dexamethasone
CRF	=	Corticotrophin-releasing factor
cGPX	=	cytosolic glutathione peroxidase
CRE	=	cAMP-responsive elements
Cu,Zn-SOD	=	Cu, Zn-superoxide dismutase
DHEA	=	Dehydroepiandrosterone
DHT	=	Dihydrotestosterone
FSH	=	Follicle-stimulating hormone
G	=	G proteins (guanine nucleotide-binding proteins)
GnRH	=	Gonadotropin-releasing hormone
GSH	=	Reduced glutathione
H	=	Tropic hormone (ACTH, LH, FSH)
hCG	=	human chorionic gonadotropin
HDL	=	High-density lipoprotein
IMM	=	Inner mitochondrial membrane
LDL	=	Low-density lipoprotein
LH	=	Luteinizing hormone
MKK3	=	Mitogen activated protein kinase kinase 3
MKK6	=	Mitogen activated protein kinase kinase 6
Mn-SOD	=	Mn-superoxide dismutase
MT	=	Menopausal transition
mtDNA	=	mitochondrial DNA
NF-κB	=	Nuclear factor κB
OMM	=	Outer mitochondrial membrane
PBR	=	Peripheral-type benzodiazepine receptor
P450scc	=	Side-chain cleavage (scc) cytochrome P450
PHGPX	=	Phospholipid hydroperoxide glutathione peroxidase
pGPX	=	plasma glutathione peroxidase
PKA	=	cAMP-dependent protein kinase or protein kinase A
PRAX-1	=	PBR-associated protein-1
R	=	Tropic hormone (ACTH, FSH, and LH) receptors
ROS	=	Reactive oxygen species
SAP	=	Steroidogenesis activator polypeptide
SCP_2	=	Sterol carrier $protein_2$
SHBG	=	Sex-hormone-binding globulin
StAR	=	Steroidogenic acute regulatory protein
StAR-related lipid transfer	=	(START) domain containing protein
StarD2	=	Steroidogenic acute regulatory protein-related transfer (START) domain protein2
TPA	=	12-*O*-tetradecanoylphorbol-13-acetate
TR	=	Thioredoxin reductase
TRE	=	Responsive element
TSPO	=	Mitochondrial translocator protein
VDAC	=	34 kDa voltage-dependent anion

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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