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Review

Leydig cell aging and the mechanisms of reduced testosterone synthesis

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ABSTRACT

In males, serum testosterone levels decline with advancing age. Though part of a complex process, this age-related decline in testosterone appears to occur, in part, due to a significant decline in the ability of aged Leydig cells to produce testosterone maximally in response to luteinizing hormone (LH). The structure of the molecular machinery responsible for the synthesis of testosterone is described, and placed in the context of Leydig cell biology. Multiple parameters related to the synthesis of testosterone by the Leydig cell have been observed to change with age. Relationships among these changes are reviewed. A discussion of potential causes of the age-related decline in Leydig cell steroidogenic capacity presents a model in which the inability of aged cells to adequately respond to hormonal stimulation results in cellular regression with concomitant decline in maximal testosterone output.

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1. Introduction

In males, the steroid sex hormone testosterone is produced by Leydig cells, situated in the testicular interstitium. The Leydig cells produce testosterone in response to hormonal stimulation by the pituitary gonadotropin luteinizing hormone (LH) (Haider, 2004). Epidemiological studies have demonstrated that with age, circulating levels of testosterone progressively decline (Harman et al., 2001). This decline is associated with alterations in body composition, diminished energy, muscle strength and physical function, reduced sexual function, depressed mood, and decreased cognitive function (Matsumoto, 2002). The age-related decline in testosterone is complex, involving factors intrinsic and extrinsic to the Leydig cell. The ability to experimentally manipulate rodent testes as well as to isolate and culture rodent Leydig cells have made it possible to address the endocrine, paracrine, cellular and molecular changes that accompany the reduced ability of Leydig cells to produce testosterone with age. This review contains an overview of Leydig cell biology and testosterone synthesis, a review of the literature investigating the age-related changes to Leydig cells, and finally a discussion of the possible causes of the age-related changes in the ability of Leydig cells to synthesize testosterone.

2. Leydig cell biology and testosterone synthesis

Adult Leydig cell testosterone production depends upon the pulsatile secretion of LH by the pituitary gland into the peripheral circulation (Ellis et al., 1983). LH binds to specific high affinity

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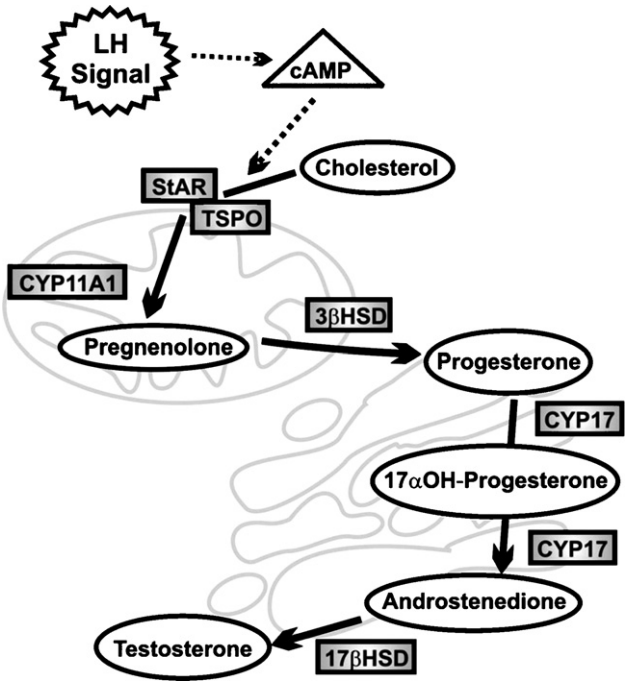

Fig. 1. Leydig cell steroidogenic pathway. In response to LH, cAMP stimulates the transport cholesterol to the inner mitochondrial membrane, where it is metabolized to pregnenolone by the cytochrome P450 enzyme CYP11A1. Pregnenolone subsequently is metabolized to testosterone by enzymes of the smooth endoplasmic reticulum: 3β hydroxysteroid dehydrogenase (3βHSD), cytochrome P450 C17 hydroxylase/17,20 lyase (CYP17) and 17β hydroxysteroid dehydrogenase (17βHSD).

receptors on the Leydig cell plasma membrane, thereby initiating a cascade of events that include LH receptor (LHR) coupling to G proteins, activation of adenylate cyclase, increased intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) formation, and cAMP-dependent phosphorylation of proteins through protein kinase A (PKA) (Ascoli et al., 2002). LH has both acute and chronic effects on Leydig cells (Ewing et al., 1983). In its acute actions, the generation of cAMP by LH results in the synthesis of testosterone (Stocco and Clark, 1996). Fig. 1 depicts the steroidogenic pathway in the rat. In response to LH, cAMP promotes the transfer of cholesterol to the inner mitochondrial membrane where it is metabolized into pregnenolone via the P450 cholesterol side-chain cleavage enzyme (P450scc/CYP11A1). Pregnenolone moves out of the mitochondria to the smooth endoplasmic reticulum where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone subsequently is converted by 17α-hydroxylase/C17-20 lyase (CYP17) to androstenedione. Finally, androstenedione is metabolized to testosterone by type 3 17β-hydroxysteroid dehydrogenase (17β-HSD3) (Payne and Hales, 2004). In parallel, chronic LH exposure regulates the expression of the major steroidogenic enzymes via cAMP (Payne and Youngblood, 1995).

2.1. LHR signaling and cAMP

The luteinizing hormone receptor is a glycoprotein member of the superfamily of G protein-coupled receptors (GPCRs) (Ascoli et al., 2002). As originally proposed by Rodbell and Gilman, the GPCR signaling module contains a receptor which recognizes an extracellular signal, an effector molecule which generates an intracellular signal, and a GTP-binding protein (G protein) which mediates signaling between the two as a molecular switch (Gilman, 1987). The G proteins are heterotrimers consisting of α, β and γ subunits. In their inactive state, the Gα subunits bind GDP. Binding of LH to the LHR activates stimulatory G proteins (G_s), which promotes the release of GDP by the α subunit and subsequent binding of GTP, conveying an "on" signal (Dufau et al., 1980). The active Gα subunit subsequently binds and activates the effector, adenylyl cyclase, which generates cAMP from the cellular ATP pool. Subsequent hydrolysis of GTP by the GTPase domain of the Gα subunit results in cessation of the hormonal signal, relegating it to an "off" position (Oldham and Hamm, 2008).

Tremendous progress in this research area over the last two decades has demonstrated that this signaling circuit is under exquisite cellular control, with multiple levels of regulation existing in living cells. Upon stimulation, GPCRs activate not only G-protein-dependent effector systems, but also initiate a series of interactions that facilitate feedback uncoupling of the receptor from its cognate G protein (Kohout and Lefkowitz, 2003). The molecular machinery responsible for desensitization of GPCRs appears receptor-specific, though desensitization effectors have been identified for the LHR. In Leydig cells, hormonal stimulation and cAMP analogs are able to desensitize the LHR to further hormonal stimulation (Cooke et al., 1992). This desensitization is recapitulated by activation of protein kinase C (PKC), though the relevance to physiological desensitization remains an open question (Inoue and Rebois, 1989). Studies of the LHR in different cellular systems has revealed that desensitization is accompanied by high affinity binding of the arrestin3 molecule (Min et al., 2002), which has been demonstrated to sterically inhibit association with cognate G protein in other receptor systems (Kohout and Lefkowitz, 2003). Moreover, desensitized LHRs have been demonstrated to possess less freedom of motion within the plasma membrane, potentially limiting their association with effector molecules (Hunzicker-Dunn et al., 2003).

2.2. Mitochondrial cholesterol transport

The primary point of post-receptor control during the acute stimulation of steroidogenesis by LH is the conversion of cholesterol to pregnenolone on the inner mitochondrial membrane by P450scc/CYP11A1 (Jefcoate, 2002). The molecular mechanism of this control involves the cAMP-dependent transport of cholesterol from cellular stores to the inner membrane of the mitochondria, where the P450scc enzyme resides (Fig. 1). This process has been an area of intense research over the last four decades, and while the precise dynamics are still under investigation, important components of this system have been identified.

The stores of cholesterol within steroidogenic cells may be supplied in the form of lipoprotein and recognized by the membrane-bound scavenger receptor class B type I (SR-B1) lipoprotein receptor (Azhar and Reaven, 2002), or in some cases synthesized de novo from acetate, involving the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase enzyme (Hou et al., 1990). Cholesterol is subsequently stored in cytoplasmic lipid droplets in the form of cholesteryl esters (Haider, 2004). Once sequestered in lipid droplets, cholesterol transport in response to hormonal stimulation can be divided into two phases. The first phase consists of mobilization of cholesteryl esters by cholesterol esterases, e.g., carboxyesterase (ES-10) and hormone-sensitive lipase (HSL), and subsequent transfer to the mitochondrial outer membrane (Shen et al., 2003; Jewell and Miller, 1998). Leydig cells may additionally derive cholesterol for steroidogenesis from their plasma membranes (Freeman, 1987). The second phase of cholesterol transfer involves the movement of cholesterol from the outer mitochondrial membrane to the mitochondrial matrix, where the P450scc enzyme resides. Of the two phases, mito-

chondrial cholesterol import has garnered most of the research attention.

The two principal proteins identified with cholesterol transport across the mitochondrial membrane are the peripheral-type benzodiazepine receptor (PBR), recently renamed Translocator Protein (18-kDa TSPO), and the steroidogenic acute regulatory protein (StAR) (Papadopoulos et al., 2006). Evidence for StAR's important role in steroidogenesis has come in part from studies of congenital lipoid adrenal hyperplasia, an autosomal recessive disease in which synthesis of adrenal and gonadal steroids is severely impaired. This disease, which is characterized by minimal steroid production, has been reported to be the result of mutations in the StAR gene (Bose et al., 1997). StAR was initially identified as a 30/32 kDa phosphoprotein that accumulates in mitochondria of Leydig cells in response to cAMP treatment and in a manner that parallels steroid formation by the cells (Epstein and Orme-Johnson, 1991) The StAR gene was cloned and the 30 kDa phosphoprotein shown to be processed from a 37 kDa cytosolic precursor protein containing a mitochondrial targeting sequence (Stocco, 2001). TSPO, in turn, is a high affinity cholesterol binding protein localized at high levels to the outer mitochondrial membranes of steroid producing tissues (Papadopoulos, 1993). Based on knowledge of its structure, TSPO has been proposed to function as a translocator of cholesterol (Papadopoulos et al., 2006). Upon addition of hCG to Leydig cells, there is a rapid increase in TSPO ligand binding that can be inhibited by a PKA inhibitor, suggesting that cAMP-induced phosphorylation of TSPO may be involved in hCG-stimulated steroidogenesis (Culty et al., 1999). Recent evidence has suggested a functional interaction between TSPO and StAR, and has led to the proposal of a hormone-induced assembly of a large multi-protein complex that facilitates intramitochondrial cholesterol movement (Liu et al., 2006).

2.3. Metabolic transformation of cholesterol by P450 and HSD enzymes and redox regulation

The biosynthetic machinery responsible for the conversion of cholesterol to testosterone in the Leydig cell involves stepwise enzymatic metabolism of sterol molecules. These enzymes fall into two principal classes: the cytochrome P450 proteins (P450) and the hydroxysteroid dehydrogenases (HSDs) (Payne and Hales, 2004). Two P450 enzymes involved in testosterone synthesis are present in the Leydig cell, the mitochondrial CYP11A1 and the microsomal CYP17, which operate in conjunction with two hydroxysteroid dehydrogenases, the mitochondrial and microsomal 3βHSD and the microsomal 17βHSD. The Leydig cell steroidogenic pathway in the rat is ordered P450scc–3βHSD–CYP17–17βHSD, which synthesize, respectively, pregnenolone–progesterone–17α-hydroxyprogesterone/androstenedione–testosterone (Haider, 2004).

While the acute mobilization of cholesterol from intracellular stores and transfer to the P450scc in the mitochondria has attracted substantial research interest in the field of steroid synthesis, a critical component in the control of steroid hormone synthesis is the regulated expression of the genes encoding enzymes needed to produce a particular steroid hormone. As noted earlier, LH stimulation of the LHR has acute and chronic effects on testosterone synthesis. Whereas the acute stimulatory phase of LH exposure involves intracellular transfer of cholesterol to the steroidogenic enzyme machinery of the Leydig cell, the chronic effect of LH exposure is to enhance the expression of the steroidogenic enzymes (Payne and Youngblood, 1995). As demonstrated in hypophysectomized animals, or in animals in which the hypothalamic–pituitary–gonadal axis has been suppressed by exogenous steroid supplementation, suppression of LH production by the pituitary results in regression of steroidogenic enzyme expression (Ewing et al., 1983). Because Leydig cells are physiologically exposed to constant LH pulses from the circulation, the chronic action of LH should not be viewed as *inducing* the expression of the steroidogenic enzymes, but rather *maintaining* high levels of active steroidogenic machinery.

Counterintuitively, hormonal- and cAMP-mediated expression of the steroidogenic enzymes is not, for the most part, directly mediated by the cAMP response element/CRE binding protein (CRE/CREB) system (Payne and Youngblood, 1995), as the promoters of several cAMP-regulated genes, such as *StAR*, lack "classical" CRE elements. Instead, cAMP appears to stimulate phosphorylation of the GATA4 transcription factor through PKA, promoting interaction with the C/EBPβ and CBP transcription factors (Viger et al., 2004). It also is known that the nuclear DNA-binding protein SF-1, belonging to the orphan nuclear receptor family (Payne and Youngblood, 1995), binds to variants of an AGGCA sequence motif present in the promoters of the P450 and HSD enzymes and is essential for enzyme expression.

In addition to genetic regulation of the steroidogenic machinery, the reactions catalyzed by the steroidogenic enzymes are regulated metabolically. The nicotinamide cofactors NAD^+/NADH and $NADP^+$/NADPH are critical for the function of the steroidogenic enzymes, as they serve as electron donors and acceptors for the hydroxylation reactions catalyzed by the P450 enzymes and the dehydrogenase and reductase reactions catalyzed by the HSD enzymes (Agarwal and Auchus, 2005; Miller, 2005). Healthy cells typically have a high reductive capacity, reflected by the ratio of reduced to oxidized forms of NAPDH (Veech et al., 1969). This reductive capacity allows the cells to carry out important cellular processes, and is critical for Leydig cell steroidogenesis, as CYP11A1, CYP17 and 17βHSD require the electrons derived from NADPH to carry out their reactions (Miller, 2005). Further contributing to the electron currency of the cell is the very large oxidized pool of NADH, which is maintained in eukaryotic cells by the persistent consumption of NADH by the mitochondrial electron transport chain (Williamson et al., 1967; Brown, 1992). NADH is also important for Leydig cell steroidogenesis, as NAD^+ serves as an electron accepting cofactor in the 3βHSD-catalyzed conversion of pregnenolone to progesterone (Thomas et al., 2003), and hormone-mediated steroidogenesis is dependent on mitochondrial respiration (Allen et al., 2006; Midzak et al., 2007).

Collectively, the relative concentrations of oxidized and reduced factors, which include not only the nicotinamide cofactors but molecular moieties such as protein thiols and chemical radicals, determine the redox state of the cell, which can be defined as the global marketplace of electron transfer reactions within the cell (Hansen et al., 2006). These transfer reactions may be termed "coupled" or "uncoupled;" coupled reactions see electron transfer between donor and acceptor near 100% (Agarwal and Auchus, 2005). During uncoupled transfer reactions, electrons are transferred form the donor to untargeted, as well as the targeted, acceptor(s). A common untargeted acceptor of uncoupled electron transfer reactions is molecular oxygen, resulting in the formation of oxygen radicals. Oxygen radicals, also called reactive oxygen species (ROS), have been demonstrated to have toxic effects on cells, and have been implicated in numerous pathologies (Dröge, 2002). Leydig cells possess an antioxidant network capable of eliminating radical species, including the enzymes superoxide dismutase, glutathione peroxidase and catalase (Luo et al., 2006). As Leydig cells possess high levels of enzymes capable of electron transfer (both microsomal and mitochondrial steroidogenic enzymes as well as mitochondrial electron transport chain complexes), the antioxidant network has been proposed to be of critical importance for protection from cellular breakdown due to the formation of oxygen radicals.

3. Aging and decline in Leydig cell testosterone synthesis: mechanisms

3.1. Primary Leydig cell defect

Leydig cells do not independently produce testosterone in response to a continuous LH signal, but rather lie in a complex feed-forward/feedback endocrine circuit involving proper functioning of the hypothalamus, pituitary gland, and circulatory system (Bremner et al., 1993). Moreover, the Leydig cells themselves reside in the complex microenvironment of the testis, which impacts Leydig cell function through paracrine factors released by the surrounding tissue (Saez, 1994). Consequently, serum testosterone is not merely a marker of Leydig cell function, but rather relies on numerous factors, any of which may contribute to the observed age-related decline in serum testosterone. For example, a hypothalamic–pituitary dysfunction that results in a decrease in amplitude of the LH secretory pulse was reported in aged male rats (Bonavera et al., 1997). Further studies with aged rats suggested that this altered LH secretion resulted from a reduction in gonadotropin releasing hormone (GnRH) release from the hypothalamus, and not a decreased pituitary responsiveness to GnRH (Bonavera et al., 1998).

The Brown Norway rat has become a well-accepted model for human reproductive aging. Importantly, the testicular tumors, pituitary tumors and obesity that characterize many rat strains, and thus make it virtually impossible to distinguish between age-associated defects and disease, occur only very rarely in Brown Norway rats. This, and the fact that the rats are long-lived (typically, the rats live to 35–40 months), have made it possible to examine the mechanisms by which age-related changes occur in the absence of confounding disease. Studies of aging Brown Norway rats have reported that the number of Leydig cells per testis remains unchanged compared to young controls (Wang et al., 1993; Zirkin et al., 1993; Chen et al., 1994). These findings suggest that functional changes to the Leydig cells, rather than their loss, accounts for the observed reduction in serum testosterone levels. In Brown Norway rats, as in the human, LH levels do not decrease significantly with age though serum levels of testosterone do decline, suggesting that although age-related functional changes may (and probably do) occur within the hypothalamic–pituitary axis, a primary gonadal lesion principally contributes to age-related testosterone decline. Consistent with this, *in vitro* studies have shown that the testes of aged Brown Norway rats and Leydig cells isolated from these testes have a reduced maximal testosterone production compared to those of young adults (Grzywacz et al., 1998; Chen et al., 2002).

3.2. Age-related changes in Leydig cell steroidogenesis

The enzymes CYP11A, 3βHSD, CYP17, and 17βHSD form a critical core of the steroidogenic pathway of Leydig cells. For example, when pituitary release of LH is suppressed by the administration of steroid, the Leydig cells regress because of lack of hormonal stimulation, losing expression of the steroidogenic enzymes (Ewing et al., 1983). Isolation of the regressed cells and then acute exposure of the cells to LH results in little steroid production, demonstrating the criticality of steroidogenic enzyme expression for the synthesis of steroids (Chen et al., 2002). In aged cells, mRNA and protein expression of the steroidogenic enzymes are significantly reduced (Luo et al., 1996, 2005). Moreover, stimulation of the cells with saturating levels of intermediates of the steroidogenic pathway (e.g., pregnenolone, progesterone, etc.) demonstrates a correlated decline in steroid product formed (Luo et al., 1996). The decreased expression levels of the steroidogenic enzymes fit well with stereological analysis demonstrating reduced absolute volume of mitochondria (Chen et al., 2002) and significant regression of the microsomal volume of aged Leydig cells (Chen et al., 1994). Thus, a substantial contribution to the age-related reduced ability of Brown Norway rat Leydig cells to produce testosterone is the result of decreased expression of the enzymes responsible for the conversion of cholesterol to testosterone; this decreased expression limits the maximal flux of steroid metabolites through the steroidogenic pathway.

However, it should be noted that age-related changes to the Leydig cell are more complex than decreased expression of the steroidogenic enzymes. As described above, it has been appreciated for some time that the principal control step of steroidogenesis is the translocation of cholesterol from stores to the CYP11A1 enzyme located in the mitochondrial matrix (Stocco and Clark, 1996; Jefcoate, 2002). This step, involving transfer of cholesterol across the two mitochondrial membranes, is dependent on the presence of two proteins, StAR and TSPO. Deletion of these two genes has been demonstrated to disrupt the synthesis of steroids (Caron et al., 1997; Papadopoulos et al., 1997), and reduction in the levels of the StAR and TSPO proteins also negatively affect the ability of Leydig cells to synthesize steroids (Hauet et al., 2005). Both StAR and TSPO mRNA and protein levels are significantly diminished with age (Luo et al., 2005; Culty et al., 2002). Moreover, it appears that the accumulation of hormonally recruited cholesterol into mitochondria is compromised in aged Leydig cells, arguing for age-related decline in mitochondrial cholesterol transfer with age (Culty et al., 2002). It thus appears that not only is the maximal ability of the Leydig cell to metabolize cholesterol to testosterone decreased with age, but the maximal ability of the cell to provide the steroidogenic pathway with cholesterol substrate is decreased as well.

As noted in previous sections, both the acute delivery of cholesterol to the steroidogenic machinery and the chronic maintenance of steroidogenic enzyme expression are regulated by cAMP production upon LH stimulation of its cognate receptor (Payne and Youngblood, 1995; Stocco and Clark, 1996). With the multitude of hormone-dependent steps altered with age, it is plausible to hypothesize that aged Leydig cells are under-stimulated by LH, even in the presence of adequate concentrations of hormone. Exposure of isolated aged Leydig cells to LH for 0–20 min, a time window relevant to the acute production of testosterone by the cells, resulted in the production of significantly less cAMP than young cells (Chen et al., 2002). Moreover, and of critical importance, stimulation of aged cells with saturating concentrations of the cAMP analog dbcAMP for 3 days restored the maximal synthesis of testosterone to the level of young controls (Chen et al., 2004b). These findings demonstrate that diminished responsiveness to LH significantly contributes to diminished maximal testosterone synthesis in aged Leydig cells.

An additional negative modulator of steroidogenesis in the aged Leydig cell may be increased expression of the cyclooxygenase-2 (COX-2) enzyme. LH, in addition to its stimulation of cAMP synthesis, promotes the release of intracellular arachidonic acid (AA). AA release in steroidogenic cells may occur intramitochondrially through the action of a mitochondrial acyl-CoA thioesterase (Acot2) (Castillo et al., 2006), and/or via classical phospholipase A2-mediation (Irvine, 1982). The released AA is subsequently metabolized by cellular lipoxygenases, epoxygenases or cyclooxygenases. Lipoxygenase and epoxygenase AA metabolites are reported to stimulate steroidogenesis through enhanced expression of the StAR protein (Wang et al., 2002). However, metabolism of AA by the COX-2 enzyme has been shown to tonically inhibit StAR gene expression and steroidogenesis (Wang et al., 2003). This may be relevant to aged rat Leydig cells; increased expression of COX-2 has been reported, and inhibition of COX-2 activity was found to enhance StAR protein levels and increase testosterone synthesis (Wang et al., 2005).

Table 1
Summary of Leydig cell parameters involved in control of testosterone synthesis and affect of aging

Physiological parameter	Affected by aging
Leydig cell number	Unaffected (Chen et al., 1994)
Circulating mean LH	Unaffected (Bonavera et al., 1997)
LH receptor number	Decreased (Chen et al., 2002)
G protein level/activity	Unaffected (Chen et al., 2004b)
Adenylyl cyclase activity	Unaffected (Chen et al., 2002)
cAMP production	Decreased efficacy; unaffected sensitivity (Chen et al., 2002)
Leydig cell mitochondrial volume	Decreased absolute; unaffected relative (Chen et al., 2001)
Mitochondrial cholesterol transport	Decreased (Culty et al., 2002)
StAR	Decreased (Luo et al., 2005)
TSPO	Decreased (Culty et al., 2002)
CYP11A1	Decreased (Luo et al., 2005)
Leydig cell endoplasmic reticulum volume	Decreased absolute and relative (Zirkin et al., 1993)
3βHSD	Decreased (Luo et al., 1996)
CYP17	Decreased (Luo et al., 1996)
17βHSD	Decreased (Luo et al., 1996)
Testosterone production	Decreased efficacy; unaffected sensitivity (Chen et al., 2002)

The mechanism of reduced acute hormonal stimulation of cAMP production by aged cells remains an open question. The formation of cAMP catalyzed by LH is initiated by LH binding to its receptor, which activates the stimulatory G protein (G_s), which in turn stimulates adenylyl cyclase to convert ATP to cAMP (Dufau et al., 1980). The LH receptor has additionally been shown to be coupled to the inhibitory G protein (G_i), which inhibits adenylyl cyclase activity (Platts et al., 1988). Experimental results do not indicate a deficiency in adenylyl cyclase with aging, as evidenced by the ability of the diterpene forskolin, which directly activates adenylyl cyclase activity, to stimulate the production of cAMP by young and aged Leydig cells equally. Upstream of adenylyl cyclase, inhibition of G_i activity by pertussis toxin did not restore the ability of the Leydig cells to produce cAMP in response to LH, arguing against a tonic G_i suppression of adenylyl cyclase involvement in the aged phenotype. However, exogenous activation of G_s through cholera toxin stimulated cAMP synthesis to equal degrees in young and aged cells. At the level of the LH receptor, radioligand binding studies revealed that the number of hormone binding sites decreased significantly with age, suggestive of decreased levels of receptor at the plasma membrane (Chen et al., 2002). This might contribute to the decreased hormone-induced cAMP synthesis, but it should be stressed that approximately 10% of LH receptor occupancy is required to elicit a biological response, indicative of reserve or "spare" receptors (Hsueh et al., 1977). In fact, LH-suppressed Leydig cells from young animals, which have even lower hormone binding than aged cells, have unaltered ability to synthesize cAMP in response to LH (Chen et al., 2002). Consequently, decreased surface levels of the LH receptor are not expected to directly contribute to decreased ability of aged cells to synthesize cAMP. Instead, the evidence points to a model in which the aged LH receptor is "uncoupled" from a functionally unimpaired G_s–adenylyl cyclase signaling circuit.

As summarized in Table 1, our current understanding of the aged Leydig cell and its ability to synthesize testosterone suggests that the ability of the cells to maximally synthesize testosterone is, first, a consequence of the decreased expression of steroidogenic enzymes; regardless of the substrate used under acute maximally stimulating conditions, testosterone production is limited by decreased maximal flux through the metabolic pathway of testosterone synthesis. Secondly, the supply of the cholesterol precursor to the steroidogenic pathway is decreased with advancing age, when compared to young controls. This decreased supply is most likely a consequence of decreased expression, and possibly function, of components of mitochondrial cholesterol transport machinery such as the StAR protein and TSPO/PBR. Thirdly, aged Leydig cells express elevated levels of the COX-2 gene, which has been demonstrated to tonically inhibit StAR protein expression and Leydig cells steroidogenesis. Pharmacological inhibition of COX-2 increases StAR expression and steroidogenesis in aged Leydig cells, suggesting COX-2 participation in the aged Leydig phenotype. Finally, the cAMP signal necessary for both maintenance of the steroidogenic capacity of the Leydig cell and the acute availability of the cholesterol precursor to the steroidogenic pathway is significantly diminished with age. This indicates that a common deficiency, inability of aged cells to acutely synthesize cAMP, underlies many of the age-related deficits in steroidogenesis observed in Leydig cells. At this point, the mechanism by which acute cAMP production is reduced remains speculative.

3.3. Mechanisms and etiology of age-related deficits

The final portion of this review discusses research that contributes to our understanding of how age-related deficits may arise in Leydig cell testosterone production. There is a general consensus that aging is a complex process, or set of processes, involving numerous causes and consequences (McClearn, 1997). However, a common theme in theories of aging involves some form of ill-defined deteriorative process. Theories of aging generally focus on basic biological processes that "cause" aging, including gene expression changes (Collado et al., 2007), metabolic generation of chemical radicals (Beckman and Ames, 1998), DNA repair (Lombard et al., 2005), and telomere shortening (Collado et al., 2007). Among these, the results of studies of Brown Norway rat Leydig cells are most consistent with alterations to redox metabolism caused by free radicals, owing to the large number of electron transfer reactions performed in Leydig cells (see above). Aging theories based on DNA replication, including replication-error and telomere shortening, do not readily apply to Leydig cells as these cells are post-replicative (Russell et al., 1995).

In 1956, Harman suggested that normal oxygen consumption inevitably results in the production of oxygen free radicals which, in turn, deleteriously modify important biological macromolecules (e.g., nucleic acids, proteins, lipids, sugars), resulting in accumulated damage over time and ultimately in death (Beckman and Ames, 1998). Over the past half century, the free radical theory of aging has been modified to encompass the realization that multiple chemical species which are not technically chemical radicals, such as aldehydes and peroxides, can contribute to oxygen-mediated alterations to cells. Thus, the oxidative stress theory of aging now proposes that a chronic state of oxidative stress exists in cells of aerobic organisms even under normal physiological conditions because of an imbalance between prooxidants and antioxidants. This imbalance leads to a steady-state accumulation of oxidative damage in a variety of macromolecules that increases during aging, resulting in a progressive loss in the functional efficiency of various cellular processes (Muller et al., 2007).

In the original concept of the free radical theory, oxygen toxicity was considered to result in structural modifications to cellular macromolecules. However, in recent years, it has become appreciated that ROS also critically serve as activators and inhibitors of classical signal transduction pathways, such as protein kinase C (PKC) signaling (Gopalakrishna and Jaken, 2000), as well as signaling mediators in their own right (D'Autréaux and Toledano, 2007). This has important implications for free radical theories of cellular dysfunction because signaling pathways, as processes capable of amplifying an initially small signal, would require little disruption

by ROS to manifest gross cellular deregulation. As a consequence, redox cycling agents and metal ions can selectively disrupt signaling pathways, and the addition of antioxidants would not protect against this disruption (Hansen et al., 2006).

As noted above, Leydig cells endogenously produce ROS from several sources, including the mitochondrial electron transport chain and the mitochondrial and microsomal cytochrome P450 enzymes (Quinn and Payne, 1984; Hanukoglu, 2006). These and other studies have suggested that ROS may desensitize the steroidogenic enzymes by creating pseudo-substrates that inhibit their activity. Moreover, cyclooxygenase and lipoxygenase enzymes have been demonstrated to be significant sources of ROS (Spiteller, 2001), and these enzyme families have recently garnered significant interest as they are expressed in Leydig cells and appear critically involved in the synthesis of steroid (Wang et al., 2003). In part, this might explain why there is reduced Leydig cell testosterone production with aging after years of continuous exposure to LH (Chen and Zirkin, 1999). Moreover, Leydig cells may be exposed to exogenous sources of ROS; testicular macrophages, which are intimately associated with Leydig cells, are considered to be sources of ROS that may impact Leydig cell function (Hales, 2002). However, though measurements of ROS-mediated reactions have been performed on testicular tissue and isolated Leydig cells, the precise source of the ROS species that give rise to peroxidation products remains uncertain (Peltola et al., 1996; Chen et al., 2001; Cao et al., 2004).

Cells possess an extended antioxidant enzymatic and non-enzymatic network responsible for the elimination of ROS and necessary for viability (Muller et al., 2007). Consequently, measured quantities of ROS and ROS-mediated chemical products must be viewed as steady-state values of production by ROS sources and elimination by antioxidants. In a number of aging systems, the steady-state levels of ROS have been observed to increase with age (Beckman and Ames, 1998). Similar findings have been observed in aged Leydig cells (Chen et al., 2001). Though the reason for this increased oxidative potential remains an open question, it is plausible that age-related reductions in levels of antioxidant enzymes are involved (Cao et al., 2004; Luo et al., 2006); presuming equal or increased ROS production/exposure with age, age-related decline in the antioxidant system might be sufficient to account for the increased steady-state ROS observed. It should be pointed out that a powerful criticism of the oxidative theory of aging has been that causal relationships between aging and oxidative load have yet to be established. This is especially true in mammals in which genetic and non-genetic antioxidant buffering have been shown to have little effect on lifespan extension (Muller et al., 2007). As noted above, disruption of cellular redox poise exerts disruptive effects on cellular signaling. This, too, may have important consequences for the aged Leydig cell, as signaling disruption appears to underlie many of the age-related alterations to testosterone synthesis.

As described above, disruption of cAMP signaling appears to underlie many of the age-related changes to Leydig cells and their ability to acutely produce testosterone. The LH receptor–G_s protein–adenylyl cyclase signaling circuit appears functionally intact with increasing age, though with a significant lesion in the ability of hormone-bound receptor to activate G_s protein and therefore adenylyl cyclase (Chen et al., 2002, 2004b). Based on current understanding of G protein-coupled receptor signaling, two possibilities arise to explain decreased LH receptor coupling to Gs protein (summarized in Fig. 2), both of which would be impacted by an increased cellular oxidative state. In the first model, a 'lipid peroxidation' model (Chen et al., 2002), interaction of lipid peroxides generated from cellular ROS with membrane lipids results in lipid cross-linking, thus decreasing molecular mobility in the plasma membrane. This model has the advantage of drawing on a rich body of work performed on model membrane systems demonstrating that lipid radicals form molecular cross-links that decrease lipid fluidity in the two-dimensional plane of the membrane (Wu et al., 1993; Karbownik et al., 2001). Moreover, experimental findings can be easily related to this model as (1) lipid peroxidation markers are consistently elevated with advancing age in the Leydig cell, (2) extended supplementation with the antioxidant vitamin E (α-tocopherol) delays age-related decreases in steroidogenesis, and (3) long-term vitamin E deficiency results in further decreases in steroidogenesis (Chen et al., 2005). The model has the disadvantage of relying on 'classical' models of membrane dynamics and GPCR signaling. More specifically, the model implicitly identifies the plasma membrane as uniformly 'fluid,' with lipid cross-linking decreasing fluidity either globally or locally. However, this view is in sharp contrast to recent work demonstrating that cellular membranes are complex structures marked by heterogeneous microdomains of differing 'fluidity' (Engelman, 2005), and that cells may regulate receptor signaling by maintaining receptors, G proteins and effectors in separate membrane compartments that are brought together into the same compartment upon receptor activation (Ostrom and Insel, 2004). Such compartmental movement of activated receptor has been demonstrated for the LH receptor (Smith et al., 2006). Thus, it may be misleading to base age-related conclusions on increased or decreased global membrane lipid fluidity.

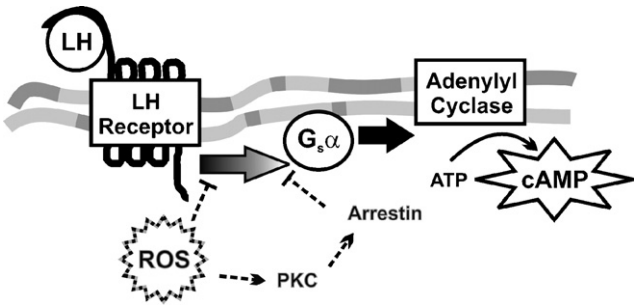

Fig. 2. Model of age-related luteinizing hormone receptor (LHR) dysfunction. LH binding to the LH receptor stimulates production of cAMP through G_s protein stimulation. However, in aged cells, this process is attenuated. Exogenous stimulation of adenylyl cyclase and G_s protein by forskolin and cholera toxin, respectively, results in equivalent cAMP production by young and aged cells, arguing for a critical lesion between the LHR and G_s in the aged cells. Increased ROS of aged cells may attenuate LHR coupling via direct modification of plasma membrane lipids or via activation of PKC signaling and receptor desensitization. Both mechanisms would limit lateral diffusion of the receptor within the membrane.

A second model of decreased LH receptor coupling to G_s protein is based on the observation that the LH receptor becomes 'desensitized' in response to persistent hormonal exposure (Cooke et al., 1992). Desensitization is characterized by decreased hormonally mediated cAMP production but G_s protein that can be pharmacologically stimulated, thus recapitulating findings in aged Leydig cells (Dix et al., 1982; Chen et al., 2004b). Uncoupling of the hormone-bound LH receptor from its downstream effectors during desensitization appears a consequence of arrestin binding, which limits molecular movement of the receptor within membranes (Min et al., 2002; Hunzicker-Dunn et al., 2003). A number of findings suggest that a redox shift to an oxidizing environment could be linked to a desensitization-type uncoupling of the receptor–cyclase interaction in aging Leydig cells. This conclusion draws upon a number of observations. Expression analysis of aged Leydig cells revealed significantly higher levels of mRNA and protein expression of protein kinase C (PKC) than in young cells (Chen et al., 2004a). PKC signaling has been identified as significantly disrupted by redox imbalance, i.e. a pro-oxidative state has been shown to

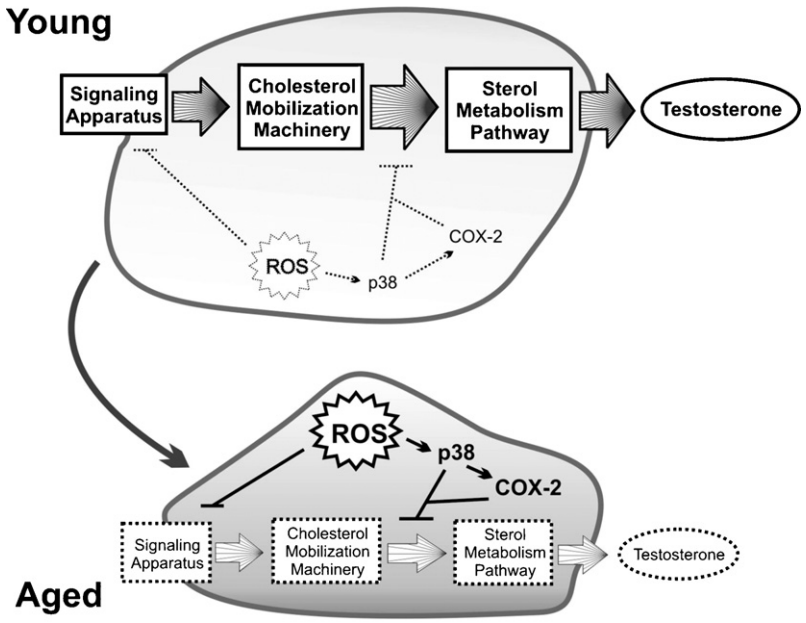

Fig. 3. Model of decreased testosterone synthesis by aged Leydig cells. As described in the text, many factors and processes involved in the synthesis of testosterone decrease with age. The evidence points to a model wherein attenuation of LH receptor signaling, potentially thorough ROS-mediated mechanisms, leads to reduced cAMP synthesis and consequent regression of the steroidogenic machinery. In addition, based on studies of adrenal cells, it seems plausible to speculate that oxidative stress signaling through the p38 MAPK cascade and elevated COX-2 expression may synergistically repress cholesterol flux. Thus, the aged Leydig cells retain their ability to be stimulated by LH, but the efficacy of LH stimulation is significantly diminished.

stimulate activation of PKC in multiple cell types (Gopalakrishna and Jaken, 2000). In turn, PKC activation has been demonstrated to uncouple the LH receptor from adenylyl cyclase in Leydig cells, consistent with observations in aged cells (Cooke et al., 1992; Inoue and Rebois, 1989). Similar observations have been reported recently in kidney cells, where oxidative stress has been found to uncouple the dopamine receptor from adenylyl cyclase-mediated cAMP synthesis in a PKC-dependent manner (Asghar et al., 2006). Moreover, reduction of oxidative stress with antioxidants was found to alleviate aged dopamine receptor desensitization (Fardoun et al., 2006), consistent with vitamin E findings with Leydig cells (Chen et al., 2005).

ROS may play an additional role in steroidogenesis inhibition through alteration in stress response signaling in cells. A well-characterized consequence of oxidative stress is activation of the mitogen-activated protein kinase (MAPK) cascade, including the p38 and c-Jun NH2-terminal kinases (JNK) (Dröge, 2002). Recent studies have reported oxidative stress-related p38 MAPK activation in both aging rat adrenal and Leydig cells (Abidi et al., 2008a,b). Intriguingly, pharmacological inhibition of oxidative stress and p38 MAPK signaling in aged adrenal cells resulted in decreased p38 MAPK phosphorylation and increased steroid synthesis by the cells (Abidi et al., 2008a). This supports the possibility of a causal impact of ROS and p38 MAPK on the decline in steroidogenesis in aged steroidogenic cells.

These observations may have importance for understanding the relevance of elevated COX-2 to age-related reductions in Leydig cell steroidogenesis. COX-2 expression in Leydig cells is stimulated by LH and cytokines (Chen et al., 2007). Cytokine expression in the testis increases with age (Jung et al., 2004), and cytokines have been shown to have inhibitory effects on Leydig cell steroidogenic enzyme expression (Diemer et al., 2003). However, the sensitivity of aged Leydig cells to cytokines is not known, and therefore it is not clear whether cytokines in fact have a role in the age-related reduction in Leydig cell testosterone production. Moreover, it is well established that the sensitivity of aging Leydig cells to LH signaling is blunted (Chen et al., 2002, 2004b). However, even in the absence of cytokine or LH stimulation, COX-2 can be induced by oxidative stress, an induction reliant on p38 MAPK signaling (Nakamura and Sakamoto, 2001; Kumagi et al., 2004; Yang et al., 2006; Abidi et al., 2008b). Though these findings have not been shown in aging Leydig cells, it seems plausible to speculate that the increased oxidative environment of aged Leydig cells may result not only in relative insensitivity to LH stimulation, but also tonically suppress StAR expression and steroidogenesis via p38 MAPK activation and elevated COX-2 expression (Fig. 3).

4. Summary and conclusions

Fig. 3 presents a model of the aged Leydig cell described in this review. Leydig cell testosterone synthesis is divided into three functional modules: (1) the steroidogenic machinery, consisting of the P450 and HSD enzymes responsible for the conversion of cholesterol to testosterone, as well as accessory enzymes and cofactors; (2) the cholesterol transfer apparatuses, including the enzymes responsible for hydrolysis of stored cholesteryl esters, movement of cholesterol to the mitochondria, and transfer of cholesterol across the double mitochondrial membranes by the StAR–TSPO transfer complex; and (3) the LH receptor–adenylyl cyclase signaling circuit. In the described model of the aged Leydig cell, ROS produced by endogenous cellular processes either physically disrupts membrane lipids or inappropriately activates the cellular desensitization machinery, resulting in attenuation of LH receptor signaling to adenylyl cyclase with concomitant decreases in maximal levels of cAMP. This attenuation of cAMP production has short-term effects on the intracellular transfer of cholesterol to the mitochondria and longer-term effects on the metabolism of cholesterol to testosterone. Moreover, inhibition of steroidogenic processes is reinforced by activation of stress-response signaling by ROS through the p38 MAPK cascade and increased expression of the COX-2 enzyme. Ultimately, there is a reduction in the maximal steroid output by aging Leydig cells. Thus, aging Leydig cells retain

their ability to be stimulated by LH, but the efficacy of LH stimulation is significantly diminished (Liao et al., 1993; Chen et al., 2002; Keenan and Veldhuis, 2004). The reduced responsiveness to stimulation is a common theme in aging systems (Yeo and Park, 2002). Future studies are likely to center on investigation of intracellular (and perhaps extracellular) molecular mechanisms leading to decreased Leydig cell responsiveness. This, in turn, may potentially lead to methods by which to ameliorate the age-related decline in testosterone synthesis in humans.

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