REPRODUCTION
REVIEW

Steroidogenesis in Leydig cells: effects of aging and environmental factors

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Abstract

Serum testosterone (TS) levels decrease with aging in both humans and rodents. Using the rat as a model system, it was found that age-related reductions in serum TS were not due to loss of Leydig cells, but rather to the reduced ability of the Leydig cells to produce TS in response to luteinizing hormone (LH). Detailed analyses of the steroidogenic pathway have suggested that two defects along the pathway, LH-stimulated cAMP production and cholesterol transport to and into the mitochondria, are of particular importance in age-related reductions in TS production. Although the mechanisms involved in these defects are far from certain, increasing oxidative stress appears to play a particularly important role. Interestingly, increased oxidative stress also appears to be involved in the suppressive effects of endocrine disruptors on Leydig cell TS production.

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Introduction

Steroid hormones regulate critical phases of development and are essential for homeostasis of key physiological functions. In the male, the Leydig cells of the testis are responsible for producing testosterone (TS), and TS is essential for spermatogenesis. TS also is important for the maintenance of secondary sexual functions. A number of longitudinal studies have shown that in most men there is a slow decline in serum levels of total and bioactive TS with aging, beginning at about age 30 years, even in the absence of disease (Harman *et al.* 2001). Decline of serum TS usually is accompanied by increased or unchanging levels of LH and increased serum levels of FSH (Zwart *et al.* 1996), suggesting that the relative unresponsiveness of the Leydig cells to LH, rather than, or in addition to, defects in the hypothalamic–pituitary axis, plays a primary role in age-related reductions in serum TS (Mulligan *et al.* 2001, Keenan *et al.* 2006). This is discussed further below. TS deficiency in the adult has consequences for the general health of individuals, including increased body fat and fatigue, and decreased muscle mass, bone density, cognitive function and immune response (Huhtaniemi 2014).

In Brown Norway rats, as in aging men, serum TS levels were shown to decrease with age (Zirkin *et al.* 1993). However, unlike the many rat strains in which age-related decreases in serum TS levels are a consequence of reduced LH levels, LH levels do not decrease in Brown Norway rats. This suggests that in this strain, as in men, there is primary hypogonadism, with age-related changes in Leydig cell steroidogenesis occurring at the gonadal level rather than secondary to hypothalamic–pituitary changes.

TS production can be affected not only by aging but also by endocrine disruptors. There now is evidence suggesting that there may be common mechanisms that explain both age-related and endocrine disruptor-induced reduced TS production. This review will emphasize research on Leydig cell steroidogenesis, focusing on the effects of aging on Leydig cell TS production. We also will discuss mechanisms in common with aging by which some endocrine disruptors also affect steroidogenesis.

Testosterone synthesis in Leydig cells

Steroidogenesis is a multi-step process that converts cholesterol into final steroid hormone products. As reviewed in Aghazadeh and coworkers (Aghazadeh *et al.* 2015), steroidogenesis consists of cholesterol mobilization from lipid droplets and/or the plasma membrane, cholesterol transport into mitochondria, pregnenolone formation in the mitochondria and subsequent conversion of pregnenolone into the final steroid products by enzymes of the smooth endoplasmic reticulum (Fig. 1). In the adult testis, Leydig cell TS production depends upon the pulsatile secretion of LH by the pituitary gland into the peripheral circulation. LH plays two essential roles in Leydig cell steroidogenesis:

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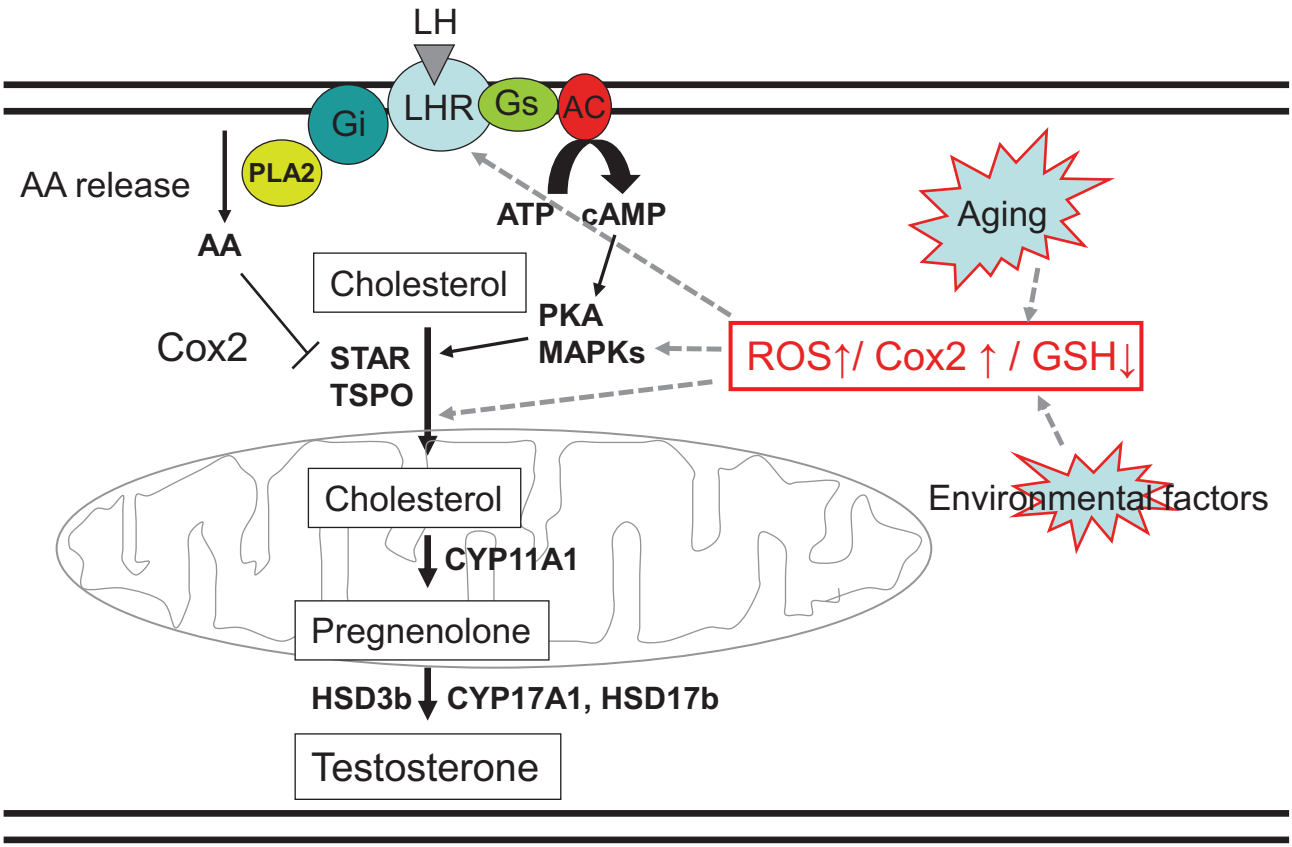

Figure 1 Leydig cell steroidogenesis. LH binds its receptor on the Leydig cell membrane. LH receptor/G protein coupling results in increased cAMP and arachidonic acid (AA) production. cAMP stimulates the mobilization and transport of cholesterol to and into the mitochondria in part by activating PKA and MAPK signaling. At the same time, AA can be converted into prostaglandin by Cox2 to negatively regulate the transport of cholesterol across the mitochondrial membranes. At the inner mitochondrial membrane, cholesterol is converted to pregnenolone by CYP11A1, and pregnenolone is converted into testosterone by enzymes in the smooth endoplasmic reticulum (HSD3b, CYP17A1 and HSD17b). Aging and environmental factors may impact steroidogenesis by affecting the intracellular redox balance in part through increased ROS production. This has significant effects on cAMP formation and/or cholesterol transport into the mitochondria, and thus on steroid formation. Steroidogenesis also might by affected by ROS-induced increases in Cox2 production and redox-sensitive MAPK activation.

(1) maintenance of optimal levels of steroidogenic enzymes (trophic regulation) and (2) mobilization and transport of cholesterol into the inner mitochondrial membrane (acute regulation).

Both the trophic and acute effects of LH are mediated by signaling pathways that begin with cAMP production (Fig. 1). LH binds to and activates G protein-coupled receptors, resulting in the activation of adenylyl cyclase, increased intracellular cAMP formation and cAMP-dependent phosphorylation of proteins through protein kinase A (PKA). The acute stimulation of Leydig cells by LH results in cholesterol transfer into the mitochondria in part through the actions of steroidogenic acute regulatory protein (STAR), translocator protein (18 kDa; TSPO) and other proteins of the transduceosome (Midzak *et al.* 2011). Cholesterol transport into the mitochondria, the rate-limiting step in steroid biosynthesis, is followed by the conversion of cholesterol to pregnenolone by the C27 cholesterol side-chain cleavage cytochrome P450 enzyme (CYP11A1) located on the matrix side of the inner mitochondrial membrane. Pregnenolone then is metabolized into TS by 3β-hydroxysteroid dehydrogenase (3b-HSD; HSD3B), 17α-hydroxylase/17,20 lyase (CYP17A1) and type 3 17β-hydroxysteroid dehydrogenase (17b-HSD3, HSD17B) in the smooth endoplasmic reticulum (Payne & Hales 2004, Aghazadeh *et al.* 2015, Beattie *et al.* 2015).

Effects of aging on steroidogenesis

Aging and declining testosterone production by Leydig cells

With aging, serum TS levels decrease in the males of a number of species, including human and rodent (Harman *et al.* 2001, Mulligan *et al.* 2001, Keenan *et al.* 2006). Studies in men have shown a reduced ability of Leydig cells to produce TS in response to LH stimulation (Veldhuis *et al.* 2005, 2012, Surampudi *et al.* 2012). In many rat strains, including Sprague-Dawley, decreased

LH levels that result from hypothalamic–pituitary changes are responsible for decreased TS formation by aging Leydig cells, and thus, for decreased serum TS (Zirkin *et al.* 1993). This is referred to as secondary hypogonadism. In Brown Norway rats, however, as in men, TS levels decrease with age despite unchanging LH and increasing FSH levels (Zirkin *et al.* 1993, Chen *et al.* 1994, 1996, 2015*a*, Beattie *et al.* 2015). Some years ago, we showed that when Brown Norway rat testes were perfused with maximally stimulating LH, testes from old (18- to 24-month-old) rats produced significantly less TS than testes from young (3- to 6-month-old) rats (Zirkin *et al.* 1993). Consistent with this, Leydig cells isolated from aged rats produced less TS than cells from young rats in response to maximally stimulating LH (Chen *et al.* 1994). Leydig cells in the adult testis rarely turn over, and their numbers do not change or increase with age (Wang *et al.* 1993, Chen *et al.* 1994). Consequently, the reduced serum TS levels in old rats results from the relative unresponsiveness of the Leydig cells to LH (Chen *et al.* 2002).

Among the important changes in the steroidogenic pathway that have been implicated in the reduced TS production that characterizes aging Leydig cells are reductions in cAMP production and protein kinase A (PKA) activities (Chen *et al.* 2002, Liao *et al.* 1993). LH binding to LH receptors initiates a cascade of events that include receptor coupling to Gs proteins, activation of adenylyl cyclase and ultimately increased cAMP production and activation of cAMP-dependent PKA (Fig. 1). Signaling through cAMP/PKA is essential for the expression of the downstream steroidogenic proteins and enzymes of the mitochondria and smooth endoplasmic reticulum (Payne & Hales 2004). Reduced numbers of LH receptors or their reduced coupling efficiency to G-protein affects cAMP production and also affects other signaling cascades including arachidonic acid/cyclooxygenase-2 (COX-2) (Wang *et al.* 2005, Castillo *et al.* 2006) and mitogen-activated protein kinase (MAPK) (Tai & Ascoli 2011). cAMP, in response to LH, stimulates the transport of cholesterol into the mitochondria (Aghazadeh *et al.* 2015). Although the culture of old cells with LH was found to result in TS production significantly below that of LH-stimulated young cells, culture with dibutyryl cAMP (dbcAMP) for 3 days resulted in increased TS production by old cells to the level of LH-stimulated young cells (Chen *et al.* 2004*a*). This suggests that relative insensitivity of old Leydig cells to LH, and consequent deficient signal transduction, contributes to, or may cause, the reduced TS that characterizes aging Leydig cells.

What is the molecular mechanism by which cAMP production is reduced with aging? Stimulation of adenylyl cyclase in old Leydig cells with forskolin, which activates adenylyl cyclase directly via by-passing the ligand and G proteins, was found to result in equivalent production of cAMP by young and old cells (Chen *et al.* 2002). Likewise, in the absence of LH, direct activation of Gs protein by cholera toxin stimulated cAMP synthesis in old cells to young levels, and inhibition of the inhibitory G protein (Gi) by pertussis toxin did not restore the ability of the aged Leydig cells to produce cAMP at high levels in response to LH (Chen *et al.* 2002). These observations suggest that G protein and adenylyl cyclase deficiencies are unlikely to cause aged cells to produce less cAMP than young cells. The number of LH-binding sites was shown to decrease significantly with age, indicating reduced plasma membrane LH receptor numbers (Chen *et al.* 2002). However, it has been known for some time that only 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, Leydig cells from young, LH-suppressed rats, which have even lower numbers of LH receptors than aged cells, nonetheless were found to have an unaltered ability to synthesize cAMP in response to LH (Chen *et al.* 2002). These results, taken together, lead to the conclusion that LH receptor-G protein coupling deficiency is likely to be responsible, at least in part, for reduced cAMP production by aged Leydig cells. It also has been suggested that increased cAMP degradation may play a role (Sokanovic *et al.* 2014). In rodents, Leydig cell cAMP is metabolized by phosphodiesterases (PDE4a, PDE4b, and PDE2a) and found to be increased in aging Leydig cells (Sokanovic *et al.* 2014). However, the extent to which these increases might reduce cAMP levels in aged cells is unclear.

In addition to its effects on cAMP production, LH stimulation also results in arachidonic acid release in Leydig cells (Fig. 1) (Wang *et al.* 2005, Castillo *et al.* 2006). The released arachidonic acid is metabolized by cellular lipoxygenases, epoxygenases or cyclooxygenases (COX). The metabolites have been shown to be capable of modulating steroidogenesis in part by affecting STAR protein expression (Wang *et al.* 2005). In MA-10 Leydig cells and rat primary Leydig cells, inhibition of COX-2 activity resulted in significantly increased TS production, suggesting that COX-2 can negatively affect steroidogenesis (Wang *et al.* 2005, Chen *et al.* 2007). In aging Leydig cells, COX-2 message and protein levels have been shown to be elevated relative to their levels in cells from young rats (Wang *et al.* 2005, Chen *et al.* 2007), suggesting the possible involvement of COX-2 in age-related reductions in TS production (Wang *et al.* 2005, Chen *et al.* 2007). This is supported by the observation that long-term treatment of aged rats with COX-2 antagonist can partially reverse reduction in serum TS levels (Wang *et al.* 2005). These findings suggest the possibility that COX2 may also be involved in the age-related decline in TS production.

Members of the MAPK signaling family, including ERK and p38, also have been implicated in age-related reductions in steroidogenesis (Fig. 1) (Abidi *et al.* 2008*a*, Sokanovic *et al.* 2014). LH has been shown to

increase ERK1/2 phosphorylation (Tai & Ascoli 2011). Inhibition of ERK1/2 phosphorylation can significantly block the effect of LH stimulation, suggesting that ERK1/2 in part mediates LH function. Age-related modulation of the p38 MAPK signaling pathway has been shown in adrenal and Leydig cells to be associated with reductions in steroidogenesis elicited by prooxidants (Abidi *et al.* 2008*a*,*b*), and reductions in steroidogenesis can be partially prevented by inhibition of p38 (Chen *et al.* 2010). In a recent study, it was found that the oxidant-sensitive p38 MAPK can inhibit cAMP-induced steroidogenesis in part by repressing STAR (Zaidi *et al.* 2014), suggesting that age-related Leydig cell dysfunction may be related to changes in both MAPK and cAMP signaling (Sokanovic *et al.* 2014).

Cholesterol synthesis, mobilization and mitochondrial transport

In addition to the reductions in LH-stimulated cAMP in aged Brown Norway rat Leydig cells, there are reductions in STAR, TSPO, the mitochondrial enzyme CYP11A1 and downstream steroidogenic enzymes of the smooth endoplasmic reticulum (Luo *et al.* 1996, 2001, 2005, Culty *et al.* 2002). Although the mitochondrial and smooth endoplasmic reticulum steroidogenic enzymes are reduced in aging Leydig cells, their levels nonetheless are sufficient to support high levels of steroid production if enough cholesterol is translocated into the mitochondria and thus is available to CYP11A1 of the inner mitochondrial membrane (Culty *et al.* 2002). This suggests that defects in cholesterol import into the mitochondria might underlie the differential steroidogenic abilities of young vs old Leydig cells and may ultimately be responsible for the reduced TS formation in response to LH that characterizes the old cells. Cholesterol translocation involves mobilization of cholesterol and its transport to the outer and then the inner mitochondrial membrane where CYP11A1 resides (Aghazadeh *et al.* 2015, Venugopal *et al.* 2016).

The precise location of the intracellular cholesterol utilized in steroid formation is uncertain. Studies have shown that cholesterol utilized in steroidogenic cells derives in part from lipoprotein in the circulation that is imported by membrane-bound scavenger receptor class B type I (SR-B1) lipoprotein receptor (Azhar & Reaven 2002) or is synthesized de novo within the cells from acetyl-CoA. Cholesterol may be stored in cytoplasmic lipid droplets in the form of cholesteryl esters. A recent study reported that the plasma membrane is a major source of cholesterol for steroidogenesis (Venugopal *et al.* 2016). In response to hormonal stimulation, cholesteryl esters are converted into free cholesterol by cholesterol esterases and then transferred to the mitochondrial outer membrane (Shen *et al.* 2003). Important components in this process, including SR-B1, carboxyesterase (ES-10) and hormone-sensitive lipase (HSL) are downregulated in aged Leydig cells (Chen *et al.* 2004*b*), suggesting that cholesterol import, synthesis and mobilization are all affected by aging. This is consistent with the observations that cholesterol synthesis and mobilization are reduced in Leydig cells of aged as compared to young rats (Liao *et al.* 1993).

Cholesterol translocation into mitochondria is mediated by cAMP through the proteins of the transduceosome (Papadopoulos *et al.* 2015). Translocator protein (TSPO), one such protein, comprises 2% of the outer mitochondrial membrane proteins of young Leydig cells. TSPO is reduced significantly in old Leydig cells (Culty *et al.* 2002). Incubating old Leydig cells with LH plus a specific TSPO drug ligand resulted in the restoration of TS production to the high levels of LH-stimulated young cells (Chung *et al.* 2013). In complementary *in vivo* studies, administering TSPO ligand to aged rats restored serum TS to the level of young rats. (Chung *et al.* 2013). A domain in the C-terminus of TSPO was characterized as a cholesterol recognition/interaction amino acid consensus (CRAC). These studies, among those conducted over many years, strongly implicate TSPO as a mediator of cholesterol translocation and thus steroidogenesis (Papadopoulos *et al.* 2015). However, recent studies of the effects of the genetic deletion of Tspo in mice have reported that TSPO knockout has little or no effect on steroidogenesis, thus calling into question the role of TSPO in cholesterol translocation (Tu *et al.* 2014). The latter results are disputed, however (Fan *et al.* 2015, Papadopoulos *et al.* 2015), and yet to be confirmed.

Several additional cytosolic proteins, including PKA, acyl-CoA binding domain-containing protein 3 (aka PAP7) and STAR may be involved in the import of cholesterol to the inner mitochondrial membrane (Papadopoulos *et al.* 2007, Miller 2013). STAR, for one, plays a significant role in steroidogenesis (Kallen *et al.* 1998, Clark 2016). When Leydig cells are stimulated by LH, STAR levels increase rapidly (Clark *et al.* 1994). In rodents, deletion of the *STAR* gene, or knockdown of the STAR protein, has been shown to suppress steroidogenesis (Lin *et al.* 1995). Impairment in the synthesis of adrenal and gonadal steroids has been shown to be associated with congenital lipoid adrenal hyperplasia in the human, a disease characterized in part by mutations of the *STAR* gene (Bose *et al.* 1997). Although the importance of STAR in mitochondrial cholesterol transfer is clear, the mechanism by which it functions remains uncertain. Early *in vitro* studies showed that STAR contains a domain (StAR-related lipid-transfer domain, or START) that binds cholesterol and may be involved in cholesterol transfer (Ponting & Aravind 1999) and thus in steroid production (Arakane *et al.* 1996). *In vivo*, the expression of a full-length STAR transgene in *STAR*-knockout mice restored adrenal and gonadal steroidogenesis (Sasaki *et al.* 2008).

These observations, and the localization of STAR at the outer mitochondrial membrane, suggest a critical role for STAR in cholesterol translocation (Clark 2016, Stocco *et al.* 2017). Moreover, recent reports of functional interaction between TSPO and STAR have led to the proposal that such interaction may be an integral part of cholesterol translocation to and into the mitochondria in steroidogenic cells (Liu *et al.* 2006, Miller 2013, Papadopoulos *et al.* 2015).

There are other potential players in steroid formation that are part of the transduceosome. These include the 14-3-3 proteins. It has been shown that 14-3-3γ is increased fourfold upon hCG stimulation and that it binds to STAR and acts as a negative regulator of steroid formation (Midzak *et al.* 2015). Each of TSPO, STAR, 14-3-3γ and perhaps other transduceosome proteins might serve as targets to regulate and thus enhance TS formation by old Leydig cells in a more physiological fashion than providing TS exogenously.

Mechanism(s) responsible for age-related changes in testosterone formation

Extrinsic factors

Cellular aging can result from changes in factors within and/or outside the target cells. Intrinsic factors, including reactive oxygen species (ROS), are discussed below. Extrinsic factors might include hormones, growth factors, oxidants and antioxidants produced by local cells or transported to the target cells via the circulation. Extrinsic factors have been shown to contribute to the aging of cells of the immune system (Badowski *et al.* 2014), skin (Lephart 2016) and muscle (Cannon 1998), among others, and to affect Leydig cells as well. For example, after the elimination of Leydig cells from the testes of young (3 months old) and aged (18 months old) Brown Norway rats by administering ethane dimethanesulfonate (EDS), a new generation of Leydig cells was restored to the aged testes by 10 weeks that produced as much TS as cells restored to the young testes (Chen *et al.* 1996). However, TS production by the newly formed Leydig in the testes of old rats was reduced to 'old' levels 20 weeks later. This was in striking contrast to the new cells formed in young testes, the TS production of which did not change in that period of time (Chen *et al.* 2015*a*). These observations suggested that factors outside the newly formed cells themselves may play important roles in their aging.

Leydig cell steroidogenic function has been shown to be affected by factors produced locally, and also at a distance via the circulatory system. Within the testis, Sertoli cell and macrophage products have been shown to have stimulatory effects on Leydig cell steroidogenesis (Haider 2004). Negative effects also have been shown. For example, hydrogen peroxide produced by macrophages may negatively impact neighboring Leydig cells over time (Hales 2002). Factors produced by the pituitary (gonadotropins), liver (IGF-1), thyroid (T3), pancreas β-cells (insulin), bone marrow (osteocalcin) and immune system reach the Leydig cells via the circulatory system. It remains to be determined how changes in these extrinsic factors affect the steroidogenic function of aging Leydig cells.

Intrinsic factors: oxidants and antioxidant molecules

Imbalance between prooxidants and antioxidants often occurs as cells age (Rebrin *et al.* 2003). Such imbalance can result in an altered redox state and an accumulation of oxidative damage to intracellular macromolecules, thus contributing to age-related functional deficits (Finkel & Holbrook 2000). Leydig cells produce ROS from several sources, including the mitochondrial electron transport chain and mitochondrial and microsomal cytochrome P450 enzyme reactions (Hanukoglu 2006). Recent studies indicate ROS production might be in response to LH, incubating Leydig cells with LH resulted in increased ROS levels as well as in DNA damage (Beattie *et al.* 2013). Leydig cells from aged rats produce significantly more reactive oxygen than cells from young rats, and this occurs despite reduced mitochondrial volume (Chen *et al.* 2001). Aging of Leydig cells also is accompanied by the reduced expressions of key enzymatic and non-enzymatic antioxidants, including Cu- Zn-SOD, Mn-SOD, glutathione peroxidase (GPX-1), microsomal glutathione S-transferase (MGST1), glutathione S-transferase (GSTM2) and glutathione (GSH), leading to increased oxidative stress and oxidative damage (e.g. lipid peroxidation) (Chen *et al.* 2001, Cao *et al.* 2004). Age-related decreases in Leydig cell antioxidant activities, gene expression and protein levels are consistent with the hypothesis that the loss of steroidogenic function that accompanies Leydig cell aging may result in part from an altered antioxidant defense system (Cao *et al.* 2004, Luo *et al.* 2006).

Experimental studies designed to go beyond correlation to establish cause–effect relationships have been reported. The long-term administration of the antioxidant vitamin E delayed age-related decreases in steroidogenesis, while long-term vitamin E deficiency had the opposite effect (Abidi *et al.* 2004, Chen *et al.* 2005). GSH is among the most significant antioxidants reduced in aging Leydig cells (Cao *et al.* 2004, Luo *et al.* 2006). To examine whether loss of GSH would affect Leydig cell TS production *in vivo*, young and old rats were treated with buthionine sulfoximine (BSO) for a week to reduce Leydig cell intracellular GSH levels. Significantly decreased TS production by both young and old Leydig cells was seen following experimental reduction of GSH, suggesting that such reductions might lead to alterations in the redox environment of Leydig cells and ultimately to decreases in TS production

(Chen *et al.* 2008). Depletion of GSH *in vitro* also reduced Leydig cell steroidogenic function, while the antioxidants vitamin E, N-tert-butyl-α-phenylnitrone and Trolox suppressed the effect of loss of GSH (Chen *et al.* 2008). A critical role of GSH in regulation of Leydig cell steroidogenesis was also suggested by a recent study reporting that the expression of γ-glutamyl transferase 5 (GGT5), an enzyme involved in GSH metabolism, was negatively correlated with Leydig cell steroidogenesis (Li *et al.* 2016).

Nrf2/Keap1 signaling is an important mechanism by which cells respond to oxidative stress (Cho *et al.* 2005, Li & Kong 2009). Normally, Nrf2 is bound to Keap1 in the cytoplasm and undergoes ubiquitination-dependent proteasomal degradation. With exposure to oxidative stress, Nrf2 dissociates from Keap1. When separated from Keap1, Nrf2 binds to the antioxidant response element (ARE) in the promoter of target genes, and thus stimulates the transcription of numerous antioxidant molecules and phase two enzymes (Cho *et al.* 2005, Li & Kong 2009). With aging, GSH and antioxidant enzymes typically decline despite elevated levels of oxidative stress. This is true of many cell types, including Leydig cells (Rebrin *et al.* 2003, Cao *et al.* 2004, Luo *et al.* 2006), and leads to a prooxidant state in aging cells. We reasoned that if Nrf2 and thus the oxidant/antioxidant environment plays a role in age-related reductions in Leydig cell TS production, the experimental knockout of Nrf2 should result in increased oxidative stress and therefore, over time, in decreased TS production. Using this genetic approach, we found that at 3 months, there was no significant difference between wild-type and knockout mice in either serum TS concentration or in the ability of Leydig cells to produce TS. However, by middle age (8 months), at which time cells from wild-type mice had not yet lost steroidogenic function, TS production by Leydig cells from the knockout mice was reduced significantly. By 24 months, both the wild-type and knockout mice had reduced serum TS and Leydig cell TS production, but with more extensive reductions in the knockouts (Chen *et al.* 2015*b*). These observations indicate that without the antioxidant master regulator Nrf2, the age-related loss of Leydig cell steroidogenic function accelerated. Moreover, the antioxidant capacity of the testis was significantly reduced in the knockout as compared to the wild-type mice at each age. With reduced expression of numerous antioxidant molecules and therefore reduced total antioxidant capacity, an increasingly prooxidant environment was seen that was reminiscent of aging, accompanied by reduced TS production by Leydig cells and reduced serum TS levels. The specific targets of an altered redox environment, and thus, the mechanism(s) by which a prooxidant environment affects Leydig cell steroidogenesis, remain unclear.

Effects of environmental factors on steroidogenesis

There is growing realization that many of the man-made chemicals released into the atmosphere have significant public health consequences, including perturbation of the endocrine system. Endocrine-disrupting chemicals (EDCs) are defined as 'substances in our environment, food and consumer products that interfere with hormone biosynthesis, metabolism or action resulting in a deviation from normal homeostatic control or reproduction' (Diamanti-Kandarakis *et al.* 2009). In this section, we will briefly discuss the effects of EDC on Leydig cell steroidogenesis, and the striking similarity in mechanisms by which these effects and those that result in age-related reduction in TS production are elicited.

Effects of EDCs on cAMP production and mitochondrial cholesterol metabolism

As indicated previously, LH-stimulated cAMP production and mitochondrial cholesterol transport and metabolism have been shown to be critically involved in age-related reductions in TS formation (Fig. 1). These same elements of the steroidogenic pathway are affected by a number of EDCs, leading to reduced TS (summarized in Table 1). Using mouse Leydig tumor cells as a model system, 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), a polybrominated diphenyl ether, was found to elicit decreases in hCG-induced cAMP levels and in the synthesis of CYP11A1 (Han *et al.* 2012). Exposure of rat primary Leydig cells to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) also was found to inhibit steroidogenesis through its effects on cAMP and CYP11A1 (Johnson *et al.* 1992, Fukuzawa *et al.* 2004). Permethrin, an insecticide, disrupted TS formation in mice by decreasing the protein and mRNA levels of STAR and CYP11A1, also indicative of an effect on cholesterol transport and metabolism (Zhang *et al.* 2007). Atrazine, a widely used herbicide, has similar effects; exposure of rats to atrazine during postnatal days 23–30 caused reduced cAMP levels in Leydig cells and decreased cholesterol transport (Pogrmic *et al.* 2009). Exposure of Leydig cells to triclosan, a chemical widely used in antimicrobial preparations, resulted in significantly decreased activity of adenylyl cyclase, synthesis of cAMP and transcriptional and translational activity of P450scc, 3β-HSD, 17β-HSD and STAR (Kumar *et al.* 2008). Lindane, an insecticide that also is used to treat lice and scabies, also was found to affect cAMP, STAR and steroidogenesis (Ronco *et al.* 2001, Saradha *et al.* 2008). Phthalates are plasticizers that are highly abundant, exhibit antiandrogenic properties and have different effects depending upon when in the lifecycle they are administered. Thus, the exposure of males to phthalates in utero can affect both Leydig cell development and adult steroidogenic function (Foster *et al.* 2001, Martinez-Arguelles *et al.* 2013). MEHP was found to inhibit steroidogenesis in

Table 1 Environmental factors affecting LHR-cAMP and mitochondrial cholesterol cascades.

Toxin	Animal cell	Age	Dose (/kgBW) duration	Effects on (LC or Testis)	Mechanisms sensitive targets	Reference
BDE-47	MA10		1 μM; 24 h	P4 production↓	cAMP↓; Cyp11a1↓	Han (2012)
TCDD	Mice	Adult	100 μg; once	Testicular TS↓	LHR↓; Cyp11a1↓	Fukuzawa (2004)
Permethrin	Mice	Adult	35 mg; 6 week	Serum TS↓ Testicular TS↓	TSPO↓; STAR↓; Cyp11a1↓	Zhang (2007)
Atrazine	Rat	Pnd23-50	200 mg; 4 week	TS+DHT productions↓	cAMP↓; LHR↓; SR-B1↓ SF-1↓; STAR↓; Cyp17a1↓; Hsd17b3↓	Pogrmic (2009)
Triclosan	Rat LCs	Adult	0.01 μM; 2 h	TS production↓	cAMP↓; STAR↓; Cyp17a1↓ Cyp11a1↓; Hsd17b3↓; Hsd3b1↓ Preventable by forskolin	Kumar (2008)
Lindane	Rat LCs	Adult	10 μg/mL; 3 h	TS production↓	cAMP↓	Ronco (2001)
PFDoA	mLTC-1/rat LCs		10 μM; 24 h	P4 production ↓ TS production↓	STAR↓	Shi (2010)
Salinomycin	Mice	Adult	3 mg; 4 week	Testicular TS↓ Testis W↓ Vesicles W↓ Epididymis W↓	STAR↓; Cyp11a1↓	Ojo (2013)
Quinalphos	Mice	Adult	7.5 mg; 7 day	Serum TS↓ Testis W↓ Vesicles W↓ Epididymis W↓ Prostrate W↓	STAR↓; Cyp11a1↓	Kokilavani (2014)
MEHP	MA-10		200 μM; 24 h	P4 production↓	cAMP↓; STAR↓	Zhou (2013)
Cobalt	MA10		100 μM; 24 h	P4 production↓	Cyp11a1	Kumar (2014)
Dimethoate	Rat	Adult	15 mg; 5 week	Serum TS↓ LH↑ FSH↑	STAR↓ Hsd17b3↓; Hsd3b1↓	Astiz (2009)
Dimethoate	Rat LCs		1 ppm; 24 h	TS production↓	STAR↓ Hsd17b3↓; Hsd3b1↓	Astiz (2012)

BDE-47, 2,2′,4,4′-Tetrabromodiphenyl Ether; Cyp11a1, Cholesterol side-chain cleavage; Cyp17a1, Cytochrome P450 17a1 (Steroid 17α-hydroxylase/17,20 lyase); DEHP, Diethylhexyl Phthalate; DHT, Dihydrotestosterone; Hsd17b3, Hydroxysteroid 17-Beta Dehydrogenase 3; LHR, Luteinizing Hormone/choriogonadotropin Receptor; MEHP, Monoethylhexyl phthalic acid; P4, Progesterone; PFDoA, Perfluorododecanoic acid; Pnd, Postnatal day; SF-1, Steroidogenic Factor 1 (NR5A1); SR-B1, Scavenger Receptor class B member 1; STAR, Steroidogenic Acute Regulatory protein; TS, Testosterone; TCDD, 2,3,7,8-Tetrachlorodibenzodioxin; TSPO, Translocator Protein.

MA-10 Leydig cells by interfering with LH-stimulated cAMP production and mitochondrial cholesterol transport/metabolism (Zhou *et al.* 2013).

Reduced testosterone formation in response to environmental factors: mechanisms

Although EDCs capable of affecting steroidogenesis are diverse in nature, an altered redox environment that leads to increased oxidative stress and thus reduced steroidogenesis are commonly seen as consequences of exposures, as they are in aging (summarized in Table 2, Mathur & D'Cruz 2011). For example, exposure of mLTC-1 cells to perfluorododecanoic acid (PFDoA) was shown to result in increased levels of ROS and hydrogen peroxide and in the inhibition of STAR expression and steroidogenesis (Shi *et al.* 2010). Exposing purified rat Leydig cells to the polychlorinated biphenyl Aroclor 1254 resulted in significant decline in the activities of enzymatic and non-enzymatic antioxidant enzymes, increase in the levels of ROS and decrease in the mRNA levels of steroidogenic enzymes (Murugesan *et al.* 2008). These changes were partially prevented by the antioxidants vitamin C and E (Murugesan *et al.* 2005). Exposure of mice to salinomycin resulted in decreased testicular TS in association with depletion of SOD and GSH and increased lipid peroxidation (Ojo *et al.* 2013). Exposure of rats to chlorpyrifos resulted in reduced TS levels, increased oxidative stress and decreased antioxidant enzymes (Sai *et al.* 2014). The testicular antioxidant defense system and lipid peroxidation were also significantly affected by quinalphos exposure (Debnath & Mandal 2000, Kokilavani *et al.* 2014).

Phthalates are another group of environmental contaminants that may affect steroidogenesis by increasing oxidative stress (Martinez-Arguelles *et al.* 2013). One of the well-established consequences of phthalate exposure is activation of peroxisome proliferator-activated receptor (PPAR)-α and -γ nuclear receptors (Hurst & Waxman 2003). Activation of PPARs not only affected lipid metabolism but also increased oxidative stress in the cells (O'Brien *et al.* 2005, Zhang *et al.* 2016). Exposure of rats to DEHP during prepuberty and puberty significantly decreased the GSH/GSSG ratio and increased TBARS levels in the testis, suggesting that phthalate is capable of

Table 2 Environmental factors affecting redox environment of Leydig cells.

Toxin	Animal cell	Age	Dose (/kgBW); duration	Effects on (LC or Testis)	Mechanisms sensitive targets	Reference
Lindane	Rat	Adult	5 mg; 12 h	StAR↓ Hsd17b3↓; Hsd3b1↓	H_2O_2↑	Saradha (2008)
PFDoA	mLTC-1/rat LCs		10 μM; 24 h	P4 production ↓ TS production↓	StAR↓; H_2O_2↑ Preventable by MnTMPyP	Shi (2010)
Aroclor1254	rat LCs	Adult	10^{-8} M; 6 h	TS production↓ Cyp11a1↓ Hsd17b3↓ Hsd3b1↓	H_2O_2↑; LPO↑ Hydroxyl radical↑; SOD↓ CAT↓; GPx↓; γ-GT↓; GR↓ GST↓; Vitamin C and E↓	Murugesan (2008)
Salinomycin	Mice	Adult	3 mg; 4 week	Testicular TS↓ StAR↓ Cyp11a1↓	LPO↑ GSH↓; CAT↓; LDH↓; SOD↓	Ojo (2013)
Quinalphos	Rat	Adult	250 μg; 3 day	Serum TS↓ Testis W↓ Germ cell loss↑	LPO↑; SOD↑; GPx ↑ CAT↓; GSH↓	Debnath (2000)
Quinalphos	Mice	Adult	7.5 mg; 7 day	Serum TS↓ StAR↓; Cyp11a1↓ Hsd17b3↓ Hsd3b1↓	SOD↓; CAT↓; GPx↓ Vitamin C↓; LDH↑ Preventable by antioxidant *Cissus quadrangularis*	Kokilavani (2014)
DEHP	Rat	Pnd21	1000 mg; 10 day	Serum TS↓	Serum LH↓; Serum FSH↓ Preventable by selenium diet;	Erkekoglu (2011)
DEHP	Rat	Pnd21	1000 mg; 10 day	GSH/GSSG↓ TBARS↑	Cu,Zn-SOD↓; CAT↑ GSH/GSSG↓; GPx4↓ Preventable by selenium diet	Erkekoglu (2014)
MEHP/ DEHP	MA-10		3 mM; 24 h	ROS↑ DNA damage↓	GPx1↓; TrxR↓; GST↓ GSH↓; p53↑ Selenium dependent	Erkekoglu (2010)
MEHP	MA-10		1 μM; 48 h	P4 production↓ TS production↓	ROS↑; Cyp1a1 network↑	Fan (2010)
MEHP	MA-10		200 μM; 24 h	P4 production↓ cAMP↓; StAR↓	ROS↑ GSH depended Vitamin E protective	Zhou (2013)
Mercury	Rat	Adult	50 ppm; 90 day	Serum TS↓ Sperm count↓	LPO↑; TBARS↑ SOD↓; CAT↓	Boujbiha (2009)
Lead	Rat	Adult	25 μg; 15 day	Serum TS↓ Hsd17b3↓ Hsd3b1↓	LPO↑; GSH↓; GST↓; SOD↓ CAT↓; GPx↓; TBARS↑ Preventable byVitamin C	Pandya (2012)
Cadmium	Rat	Adult	25 μg; 15 day	Serum TS↓ Hsd17b3↓ Hsd3b1↓	LPO↑; GSH↓; GST↓; SOD↓ CAT↓; GPx↓; TBARS↑ Preventable byVitamin C	Pandya (2012)
Cobalt	MA10		100 μM; 24 h	P4 production↓ Cyp11a1↓	ROS↑ HIF-1a activity↑ Preventable by PDTC	Kumar (2014)
Arsenite	Mice	Adult	11.5 ppm; 36 day	Serum TS↓ Hsd17b3↓ Hsd3b1↓	GSH↓ Preventable byVitamin C	Chang (2007)
BPA	R2C		0.1 nM; 24 h	TS production↓ Aromatase↑	COX2↑; PGE2↑ EP2↑; EP4↑; CREB↑ Preventable by inhibitions of PKA/ Akt/ ERK/ JNK/ p38	Kim (2010)
Dimethoate	Rat	Adult	15 mg; 5 week	Serum TS↓ StAR↓ LH↑	LPO↑; PGE2↑; PGF2α↑ COX2↑; α-tocopherol↓ Preventable byTROLOX or rofecoxib	Astiz (2009)
Dimethoate	Rat LCs		1 ppm; 24 h	TS production↓ StAR↓	LPO↑; COX2↑ Preventable byPUFA	Astiz (2012)

BPA, Bisphenol A; CAT, Catalase; COX2, Cyclooxygenase-2; CREB, cAMP Response Element Binding protein; EP2, Prostaglandin E2 receptor 2; EP4, Prostaglandin E2 receptor 4; GPx, Glutathione Peroxidase; GR, Glutathione Reductase; GST, Glutathione-S-Transferase; γ-GT, γ-Glutamyl Transpeptidase; HIF-1a, Hypoxia Inducible Factor 1, alpha subunit; LDH, Lactate Dehydrogenase; LPO, Lipid peroxidation; MnTMPyP, Manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; PGE2, Prostaglandin E2; PUFA, Polyunsaturated Fatty Acids; Ppm, Parts per million; SOD, Superoxide Dismutase; TBARS, Thiobarbituric Acid Reactive Substances; TrxR, Thioredoxin Reductase.

increasing oxidative stress and affecting the redox environment *in vivo* (Erkekoglu *et al.* 2014). In MA-10 cells, MEHP treatment resulted in increased ROS levels, while depletion of GSH by BSO pretreatment greatly exacerbated the MEHP induced-ROS production and resulted in reduced progesterone production (Zhou *et al.* 2013). These results suggest that a likely mechanism by which MEHP acts is through increased oxidative stress (Zhao *et al.* 2012).

There are metals in the environment that also have been shown to affect Leydig cell steroidogenesis, with suggestions that they may do so by increasing oxidative stress. For example, mercury affects Leydig cell steroidogenesis and has effects on the antioxidant defense system (Boujbiha *et al.* 2009). Vitamin E was shown to protect against mercury-induced toxicity in mice (Rao & Sharma 2001) and sodium selenite and/or vitamin E to result in reduced lipid peroxidation, increased superoxide dismutase, catalase and glutathione peroxidase activities and reduced histopathological lesions (Kalender *et al.* 2013). Lead and cadmium, either alone or in combination, were found to disrupt the testicular steroidogenesis and antioxidant defense mechanisms, and the administration antioxidant agents were found to reduce metal-induced oxidative stress and to provide protection against lead and cadmium toxicity (Liu *et al.* 2009, 2013, Ayinde *et al.* 2012, Pandya *et al.* 2012). Exposure of MA-10 Leydig cells to cobalt chloride resulted in an increase in the production of ROS and a decrease in progesterone production (Kumar *et al.* 2014). The adverse effects of arsenite on the male reproductive system also may be mediated by oxidative stress; the exposure of mice to arsenite reduced testicular GSH levels and increased protein carbonyl content, accompanied by decreases in testicular steroidogenic enzyme activities, and changes induced by arsenite were partially prevented by the antioxidant ascorbic acid (Chang *et al.* 2007).

In addition to modifying intracellular oxidative stress, some environmental compounds also have been shown to affect COX-2, arachidonate metabolism and MAPK signaling molecules. For example, bisphenol A (BPA) induced a decrease in TS production in rat Leydig R2C cells, associated with increased COX-2 and MAPK signaling (Kim *et al.* 2010). Dimethoate, a widely used organophosphate, was reported to reduce Leydig cell steroidogenic function in association with decreased arachidonate and increased COX-2 (Astiz *et al.* 2009, 2012).

Summary and conclusions

Leydig cell TS production decreases with aging and exposure to environmental contaminants. Although the exact mechanisms responsible for changes in steroidogenesis remain uncertain, there appear to be common causative features. Both aging and a number of EDC exposures have effects on LH-stimulated cAMP production and cholesterol transport to and metabolism within in mitochondria. Increased oxidative stress appears to be responsible for at least some of the changes in the steroidogenic pathway. COX-2 and MAPK also may be involved. In addition to intrinsic mechanisms, aging and environmental factors also may affect Leydig cell steroidogenesis through endocrine, autocrine and paracrine mechanisms. In light of increased oxidative stress that accompanies aging, it would be of particular interest to determine whether EDC exposures affect aged cells differently than young cells. As yet, this has received little attention.

Declaration of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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