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Impact of Aging on Cholesterol Transport Protein Expression and Steroidogenesis in Rat Testicular Leydig Cells

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Abstract: The current studies indicate that Leydig cells from old (24-27-Mo) rats secreted significantly less cholesterol in response to stimulators, hCG, forskolin, or Bt_2cAMP as compared to cells from young mature (5-Mo) animals. This deficiency was reversed by incubation of cells with free diffusible hydroxycholesterols, indicating that age-related decline in testosterone secretion primarily results from the reduced availability of substrate cholesterol. Aging also significantly diminished the Leydig cell mRNA levels of StAR/StarD1, StarD2 and StarD4 both under basal conditions in response to hCG stimulation. Likewise, aging decreased the mRNA levels of PBR/TSPO. These changes correlated well with the reduced accumulation of cholesterol in Leydig cell mitochondria from old animals. Our results suggest that aging caused impaired expression of key cholesterol transport proteins, StAR/StarD1, StarD4 and PBR/TSPO that resulted in inefficient delivery of cholesterol to and within the mitochondria, and subsequently reduced conversion of cholesterol to pregnenolone and decreased testosterone production.

Keywords: Steroidogenic acute regulatory protein (StAR protein), StART proteins, peripheral type benzodiazepine receptor (PBR), testosterone, cholesterol metabolism, cholesterol binding proteins, cholesterol transport proteins.

INTRODUCTION

Numerous cross-sectional and longitudinal studies have established that circulating levels of testosterone decline with advancing age in men [1-7] 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, osteosporosis, insulin resistance, diabetes and increased cardiovascular risk [8-10]. Likewise, advancing age in experimental animals is also associated with profound changes in the synthesis and secretion of testosterone by the testicular Leydig cells [10-16]. In rat Leydig cells, age-related loss of testosterone production appears not to be a function of reduced gonadotropin receptors, cAMP production, or a defect in the steroidogenic pathway enzymes, and major alteration occurs distal to cAMP generation and hormone receptor interactions [14]. Work over the past several years from this laboratory has suggested that a defect in the way Leydig cells process intracellular steroid precursor cholesterol is linked with the observed decline in sterodogenesis, and that the basic problem for the aging cells is that an adequate amount of cholesterol is not available for the first step in cholesterol metabolism, i.e., precursor cholesterol is not appropriately transported to and from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where cholesterol is metabolized to pregnenolone [14,17].

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The rate-limiting step in steroid hormone biosynthesis in steroidogenic tissues is the translocation of cholesterol from the OMM to the IMM where it is converted to pregnenolone by the cholesterol side chain cleavage enzyme, P450scc (CYP11A1), thus initiating the synthesis of steroid hormones [18-22]. Over the years, a number of proteins including sterol carrier protein-2 (SCP-2) [23, 24], steroidogenesis-activator polypeptide (SAP) [25, 26], polypeptide diazepam binding inhibitor (DBI) [27, 28], des-(Gly-Ile)-DBI (endozepin) [29, 30], peripheral-type benzodiazepine receptors (PBR) [28, 31] and steroidogenic acute regulatory (StAR) protein [32] have been implicated in cholesterol transport to and within the mitochondria. Among these, StAR and PBR have gained wider acceptance in recent years, and are now believed to be major mediators of cholesterol flow from OMM to IMM for steroid synthesis [33, 34]. StAR, a hormonally regulated 37-kDa protein, acts on the outer mitochondrial membrane to facilitate the cholesterol transport to the inner mitochondrial sites where P450scc is localized [35]. StAR protein is also a prototype of the large family of StAR-related lipid transfer (START) domain containing proteins (StarD proteins), which are implicated in intracellular lipid transport, lipid metabolism, and cell signaling events [36, 37]. Of the fifteen mammalian START domain proteins identified so far [36, 37], only StAR/StarD1, StarD3/MLN64, StarD4, StarD5 and StarD6 exhibit steroidogenic potential [38, 39]. Pharmacological and genetic studies also suggest that PBR is required for mitochondrial cholesterol import [33, 40]. PBR, now referred to as translocator protein (TSPO), is a component of the multimeric 140-200-kDa complex located on the OMM especially at the OMM-IMM contact sites [40]. The complex consists of 18-kDa PBR or TSPO itself (and its polymorphic forms), the 34 kDa volt-

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age-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) [40]. Recent evidence suggests a functional interaction between StAR and PBR/TSPO [35, 40-45]. Whereas the predominant view is that StAR delivers cholesterol to PBR/TSPO, which, in turn, mediates the cholesterol transport from outer to inner mitochondrial membrane, some investigators believe that StAR removes cholesterol from the cholesterol-binding domain of PBR/TSPO and transports it to the inner mitochondrial membrane [35].

Previous studies from this laboratory [17] and others [46,47] have shown that age-related decline in Leydig cell steroidogenesis is accompanied by parallel changes in the expression of StAR/StarD1 and PBR/TSPO. The current studies were initiated to further explore these observations, and to critically evaluate the impact of aging on the expression of potential cholesterol transport proteins such as SCP2, SAP (changes in its precursor, BiP/GRP78 [48]), StarD1-6 proteins, and the key components of the PBR/TSPO complex. By using qPCR, our data indicate that mRNA expression of StAR/StarD1, StarD2 and StarD4 is significantly decreased in rat Leydig cells from old animals both under basal conditions and in response to hormonal stimulation. The mRNA levels of SCP2, BiP/GRP78 (SAP precursor), DBI, StarD3, and StarD5, however, are not affected by advancing age. We also show that aging specifically down-regulates the mRNA levels of PBR/TSPO itself, but has no effect on other key components of the PBR/TSPO complex. Based on these findings, we suggest that aging induced-impairment in Leydig cell steroidogenesis results from down-regulation of a select group of cholesterol transport proteins involved in intramitochondrial cholesterol transfer and its subsequent utilization by P450scc (CYP11A1) for steroid production.

MATERIALS AND METHODS

Animals

All experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the Department of Veterans Affairs Palo Alto Health Care System (VAPHCS). Male Sprague-Dawley rats were used for all studies. Young rats at 2-months of age were purchased from Charles River Laboratories, Inc., (Wilmington, MA), and maintained in our animal facility and used at 5-months of age (young mature, **Y**). Likewise, 23-24 month-old rats were purchased from the same vendor through a special contract and allowed to age to 24-27 months (old, **O**) in our animal facility reserved for aging studies. The animals were individually housed, fed ad libitum, and used for experiments 4h after fasting. All animals were checked for gross pathology before use. Those with visible kidney, pituitary, adrenal, testicular tumors, or other apparent defects were not used (~5% of 24-27 month-old rats). In general, two pooled testes obtained from the same animal were used per experiment.

Isolation and Purification of Testicular Leydig Cells

Testicular interstitial cells containing 10-15% Leydig cells were isolated by collagenase digestion of de-capsulated testes obtained from 5- and 24-27-month old rats. Highly purified (average 85-90%) Leydig cell preparations were obtained by subjecting interstitial cell suspensions to isoosmotic Percoll density gradient centrifugation as previously described [14].

Measurement of Hormone-Stimulated Testosterone Production by the Primary Leydig Cells

Freshly isolated Leydig cells from young (5-Mo) and old (24-27-Mo) were used for the measurement of steroidogenesis. Cell samples were incubated with ± hCG (10 ng/ml), ± forskolin (50 μM), ± Bt_2cAMP (2.5 mM), ± 20α-hydroxycholesterol (10 μM), or ± 22(R)-hydroxycholesterol (10 μM) at 37°C for 3 hours, and samples of incubation medium were assayed for testosterone by direct radioimmunoassay of testosterone produced per microgram of DNA and are expressed as the mean ± SE of duplicate determination of four different Leydig cell preparations derived from four individual rats.

RNA Isolation

Total RNA was isolated from young and old Leydig cells (pre-incubated with or without 10 ng/ml hCG) using TRIzol® (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The purity and concentration of RNA samples were determined by following absorbance (A_{260}/A_{280}) ratios. The integrity of the purified RNA samples was confirmed by 1.2% formaldehyde-agarose gel electrophoresis.

Measurement of mRNA Levels by Real-Time Quantitative PCR (qPCR)

The specific primer sets used to detect the mRNA expression of StAR/StarD1, StarD2-D6, PBR/TSPO, VDAC1-3, BiP/GRP78, gp96/GRP94, SCP-2 and SCP-x (encoded from independent promoters of the SCP-x/SCP-2 gene [49]), DBI and 28S rRNA were developed using Primer Express software (Applied Biosystems) according to the recommended guidelines based on sequences accessed through GenBank. Table **1** shows the primer sequences for the real-time quantitative PCR assay used. Reverse transcription (RT) was performed with murine leukemia reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) using both random hexamer and d(T)17 primers [45]. To eliminate any residual genomic DNA, total RNAs were treated with DNase I (Invitrogen) for 45 min (2 units/1 μg of RNA) and then extracted with phenol-chloroform before reverse transcription [50]. Amplification of cDNAs was performed with an ABI Prism 7900 system according to the manufacture's instructions. Each sample consisted of 1 μl of cDNA, 4 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 500 nM of each sense and antisence primer, 2 μl of 10X PCR buffer, TaqMan polymerase, and SYBR® Green in a final volume 20 μl [51]. As an internal quantitative control for gene expression, 28S rRNA gene expression was also determined. The StAR/StarD1, StarD2-D6, PBR/TSPO, VDAC1-3, BiP/GRP78, gp96/GRP94, SCP-2, SCP-x, and 28S rRNA gene expression of all cDNA samples was determined by fluorescence from SYBR Green. The final data were normalized to 28S rRNA, and the ratios of StAR/StarD1, StarD2-D6, PBR/TSPO, VDAC1-3, BiP/GRP78, gp96/GRP94, SCP-2 and SCP-x to 28S rRNA represented the normalized relative levels of each mRNA. Each sample was measured in triplicate plus a control without reverse transcriptase.

Mitochondrial Cholesterol Levels of Isolated Leydig Cells from Young and Old Rats

Aliquots of Leydig cells from young (5-mo) and old (24-27-mo) were incubated for 90 min without (control) or with hCG (10 ng/ml), or with hCG + aminoglutethimide (0.4 mM) or hCG + cycloheximide (0.1 mM). Subsequently, mitochondria were isolated using the hybrid Percoll/Metrizamide gradient centrifugation of Madden and Storrie [52]. The activity of cytochrome c-oxidase, a mitochondrial marker was enriched about 20-fold. Only trace amount of activities of lysosomal, endoplasmic reticulum, Golgi, peroxisomal, plasma membrane and cytosolic markers were found in various preparations [53]. The mitochondrial preparations were analyzed for cholesterol content fluorometrically [54].

Statistical Analysis

The results are expressed as the Mean ± SE. The data were analyzed by two-way analysis of variance (ANOVA). Subsequently, the Student's *t*-test was performed to determine the significance of the differences between the mean values obtained from young and old rats. A p value of <0.05 or less was considered to be statistically significant. The

Table 1. Real-Time qPCR Primer Sequences

mRNA Targets	Accession #	Primer
rANC1	D12771	5'-TGTCCAGGGCATCATCATCTACAGAGC-3' 5'-GGCTGTCACACTCTGGGCAATCATC-3'
rSCP-x	NM_138508	5'-TCCAAAGCTGTGGAAATTGTGGCA-3' 5'-TAGCACTTCCTGGCAGCTTCTTTACTCA-3'
rSCP-2	M34728	5'-ACTGGGGCTCTGTCCAGAAGGACAA-3' 5'-CAGTGGGTGTCCCTTGGAGATGA-3'
rVDAC1	NM_031353	5'-AACATGGCTGTGCCTCCCACATA-3' 5'-CAATCCATTCTCGGACTTCGTTTTC-3'
rVDAC2	NM_031354	5'-ATGGCTGAATGTTGTGTACCGGTAT-3' 5'-CATCCAGCTTTACCAACCCAAAAC-5'
rVDAC3	NM_031355	5'-GTTGACATAGATTTTTCTGGACCGACC-3' 5'-CTGACACAGTTTGGATTTGGCTGT-3'
rPBR/rTSPO	NM_012515	5'-TCCTGCTTTCATGACCATTGGGC-3' 5'-ACAACTGTCCCCGCATGGGACTTAG-3'
rDBI	NM_031853	5'-GCCTCAAGACTCAGCCAACTGATGAA-3' 5'-AGTCCCACTTGGCTTTGCCCTT-3'
rBiP/GRP-78	NM_013083	5'-GAAGAAATTGTTCAGCCAATTATCAGCA-3' 5'-GCAGATCAGTGCACCTACAACTCATC-3'
rGRP-94/gp96	DQ139270	5'-GGTCGTGGAACAACGATTACTCTTGTC-3' 5'-GCTACTCCACACATAGATGGGAAAGTTG-3'
rStAR/StarD1	AY736357	5'-ACTTGGTTCTCAACTGGAAGCAACA-3' 5'-TGGCACCACCTTACTTAGCACTTCAT-3'
mStarD2	AF151639	5'-ATCGAGAGCGATGGCAAGAAGG-3' 5'-TCCCGGTCTCCTCCTTAGGTTTT-3'
mStarD3	BC003313	5'-CCCATTGTCTCTTTCGTCCTGG-3' 5'-CGGAGAACAGCAGGGGTCCA-3'
mStarD4	AF480297	5'-CACTCTGATCCAGTATCACAGCATCGAA-3' 5'-TTGACCACGTCATCCATAACTCCTTG-3'
mStarD5	AF480302	5'-GCGTCGGGCTGGAAGAAGTGT-3' 5'-GAGGCCCGAAGCAACTGGCT-3'
mStarD6	BC061022	5'-CTCTGACTTCCTCTTCAAACATGATCAC-3' 5'-CGATAAAGTCTCTAGGGGAAATTGAGC-3'
28S RNA		5'-GCAGGGCGAAGCAGAAGGAAACT-3 5'-TGAGATCGTTTCGGCCCCAA-3'

rANC1 or ANT1, Adenine nucleotide transporter 1; SCP-X, Sterol carrier protein-X; SCP-2, Sterol carrier protein-2; rVDAC1, 2 and 3, Voltage-dependent anion channel 1, 2, and 3; rPBR/rTSPO, Rat peripheral benzodiazepine receptor/rat translocator protein; rDBI, Polypeptide diazepam binding inhibitor; GRP-78, Glucose-regulated protein-78; GRP-94, Glucose-regulated protein-94.

program used for statistical analysis was GRAPH PAD PRISM™, Version 4.0 (Graph Pad Software, San Diego, CA).

RESULTS

Aging and Testosterone Secretion by Isolated Leydig Cells

To examine the effects of aging on testicular steroidogenesis, rat Leydig cells were isolated from 5-Mo (Y) and 24-27-Mo (O) rats and evaluated for their ability to synthesize and secrete testosterone *in vitro* in response to various modulators. The results presented in Fig. (**1**) demonstrate that under basal conditions, testosterone production was comparable between young (12.6 ± 3.2 per µg DNA) and old (10.2 ± 3.0 per µg DNA) Leydig cells. However, maximal hCG induced testosterone production diminished significantly in response to aging, with mean testosterone of 484 ± 95 ng in the Leydig cells from young rats *vs* 107 ± 28 ng in Leydig cells from old rats (p <0.005). Likewise, forskolin or Bt_2cAMP-induced testosterone production also significantly decreased in response to advancing age.

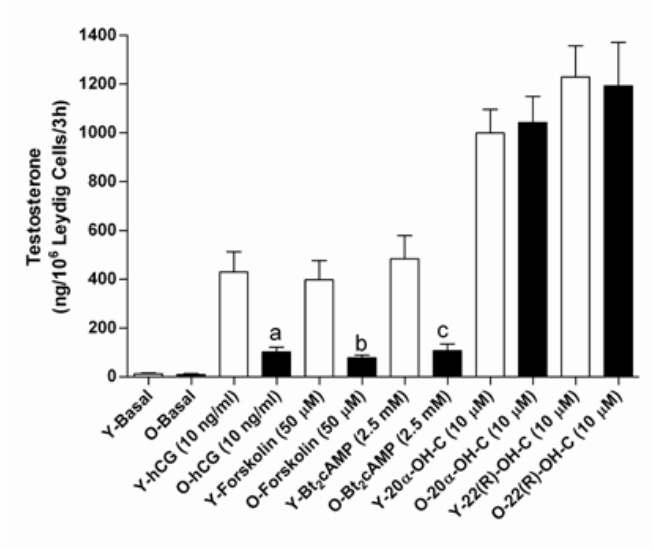

Fig. (1). hCG-, forskolin-, Bt_2cAMP- and hydroxycholesterol-stimulated testosterone secretion by Leydig cells from young (5-Mo) and old (24-27-Mo) rats. Aliquots of Percoll purified Leydig cells from young (**Y**, 5 Mo) and old (**O**, 24-27 Mo) rats were incubated without (basal) or with hCG + IBMX, forskolin + IBMX, Bt_2cAMP (2.5 mM), 20 α-OH-C (10 µM) or 22(R)-OH-C (10 µM) for 3h. At the end of incubation, medium plus cell samples were analyzed for testosterone production by radioimmunoassay. Results are Mean ± SE of four separate experiments. IBMX, 3-isobutyl-1-methylxanthine; 20 α-OH-C, 20 α-hydroxycholesterol; 22(R)-OH-C, 22(R) hydroxycholesterol.

a = p <0.005; b = p <0.002; c = p <0.001.

In order to demonstrate that aging inhibits testicular steroidogenesis by interfering with cholesterol transport to mitochondria, we also examined the aging effect on hydroxycholesterol-supported testosterone production in Leydig cells. Hydroxycholesterols such as 20α-hydroxycholesterol and 22(R) hydroxyl-cholesterol are freely transported to mitochondria, and the extent of their conversion is indicative of the functional efficiency of steroidogenic pathway, and also the extent of the mitochondrial cholesterol that may be available for steroid production [14,17]. Both young and old Leydig cells showed robust but similar secretion rates of testosterone when challenged with either 20α-hydroxycholesterol or 22(R) hydroxyl-cholesterol. These results provide additional support to the notion that age-related decline in testosterone production results primarily from inefficient cholesterol transport to and within the mitochondrial membranes.

Effects of Aging on the mRNA Expression of StarD Proteins

To uncover the aging effect on the gene expression of StarD cholesterol transport proteins, StarD1-6, and correlate changes with steroidogenic responses, mRNA samples from

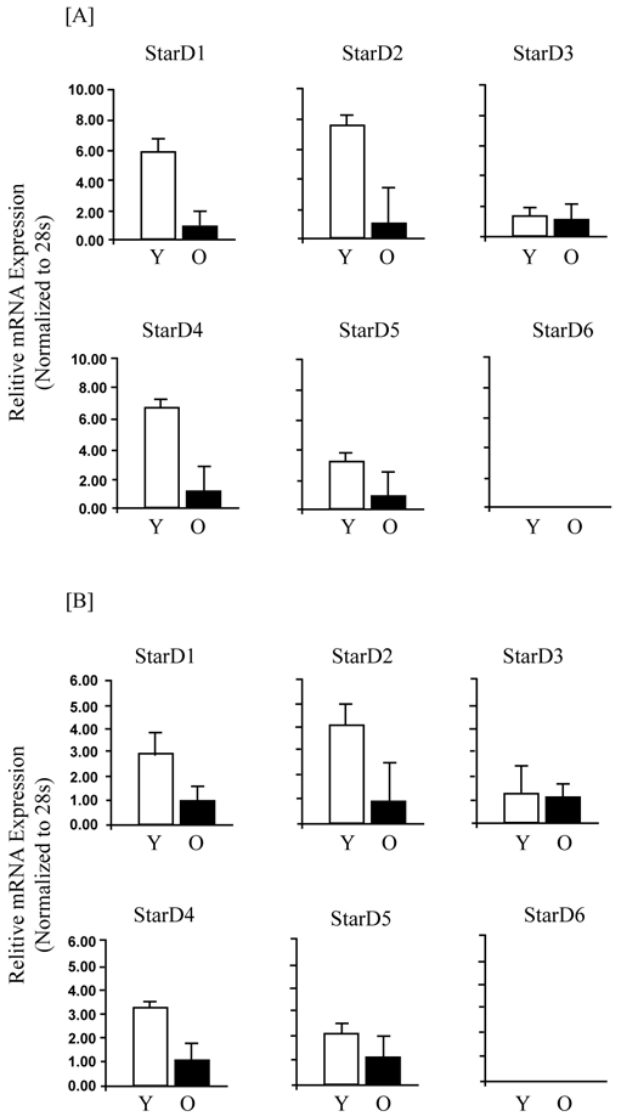

Fig. (2). Real-time quantitative PCR analysis of basal and hCG-stimulated mRNA expression of StarD family of proteins in Leydig cells from young (5-Mo) and old (24-27-Mo) rats. Aliquots of Percoll purified Leydig cells from young (Y, 5 Mo) and old (O, 24-27 Mo) rats were incubated without (basal) or with hCG + IBMX for 3h. Subsequently, isolated RNA samples were analyzed by real-time quantitative PCR. [A] Basal; [B] stimulated (hCG + IBMX). Results are Mean ± SE of 3 separate experiments.

Leydig cells of young and old rats were analyzed by real-time quantitative PCR both under basal conditions and in response to hormonal stimulation. The mRNA levels of StAR/StarD1 (p <0.001), StarD2 (p <0.04) and StarD4 (p<0.005) in basal Leydig cells from old rats were clearly lower than that of cells from young animals (Fig. **2**). In contrast, basal expression levels of StarD3 and StarD5 mRNAs remained unchanged during aging (Fig. **2**). No StarD6 mRNA expression was detected in either of the two cell types. Similar to basal levels, the hCG-stimulated mRNA levels of StAR/StarD1 (p <0.01), StarD2 (p <0.05) and StarD4 (p<0.02) were also reduced with advancing age. Finally, neither aging nor hCG had any effect on the mRNA levels of StarD3 and StarD5.

Age-Related Changes in the Constituent Proteins of the PBR/TSPO Contain Mitochondrial Macro-Molecular Complexes

The PBR/TSPO mitochondrial macromolecular complex is known to contain polymers of PBR/TSPO (18, 36 and 54 kDa), the VDAC, ANC, a 10-kDa protein (pk 10), PRAX-1, and PAP7 [39]. To determine whether aging affects the PBR/TSPO macromolecular complexes, we measured the mRNA expression of selected constituent proteins such as PBR/TSPO itself, VDAC and ANC using RNA preparations isolated from young mature (5-mo) and old (24-27-mo) rats. As shown in Fig. (**3**), the levels of PBR/TSPO mRNA in the Leydig cells selectively and significantly decreased in old rats (p<0.005). No age-dependent changes in mRNA levels, however, were noted for either VDAC (VDAC1, VDAC2 and VDAC3 isoforms) or ANT1 (Fig. **3**). Furthermore, aging had no effect on mRNA levels of DBI, an endogenous ligand that binds PBR/TSPO.

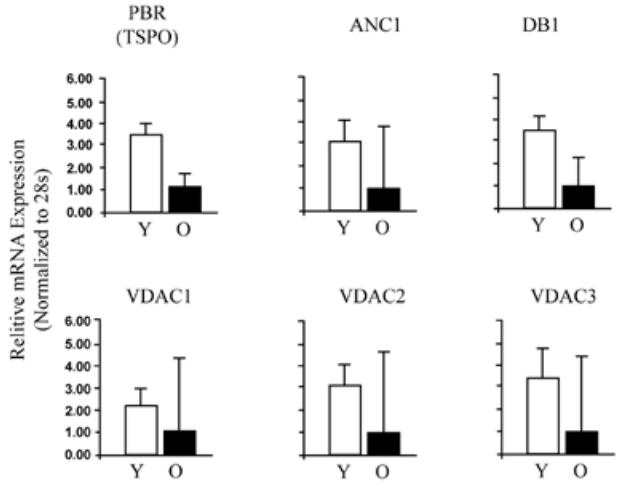

Fig. (3). Real-time quantitative PCR analysis of PBR/TSPO, ANT1, DBI and VDAC1-3 mRNA expression in rat Leydig cells isolated from young (5-Mo) and old (24-27-Mo) rats. RNA samples from Percoll purified preparations of Leydig cells from young mature (Y, 5-Mo) and old SD (O, 24-27-Mo)rats were analyzed by real-time quantitative PCR. Results are Mean ± SE of 3 separate experiments.

Aging and Leydig cell mRNA Expression of SCP-x, SCP-2, BiP/GRP-78/SAP and GRP-94 Proteins

SCP-2 and SAP are two additional cholesterol carrier molecules which have been shown to promote steroidogenesis [32] presumably by mediating the translocation of cholesterol to the inner mitochondrial membrane for side-chain cleavage by P450scc (CYP11A) and the formation of pregnenolone, the precursor steroid for the synthesis of tissue-specific steroids. SCP-2 and related protein SCP-x are encoded from independent promoters of the same SCP-x/SCP-2 gene [49]. However, while the SCP-x/SCP-2 gene is organized so that SCP-x contains SCP-2 entirely in the carboxyl-terminus (for a review, see Ref. [49]), it is still not entirely clear whether SCP-2 is formed by a distinct initiation site or by posttranslational cleavage from SCP-x [49]. Similarly, SAP was found to be nearly identical to the carboxyl-terminus of a minor heat shock protein known as BiP/GRP-78. To assess the aging effect on SCP-2 and SAP gene expression, we measured mRNA expression of SCP-2 and its potential precursor, SCP-x and SAP precursor, BiP/GRP-78, using RNA samples isolated from Leydig cells of young (5-Mo) and old (24-27-Mo) rats. As a BiP/GRP-78 control, we also measured the mRNA expression of another glucose-regulated protein, GRP-94. Fig. (**4**) demonstrates that the steady-state mRNA levels of any of these four proteins (i.e., SCP-2, SCP-x, Bip/GRP-78 and GRP-94) were not affected by aging.

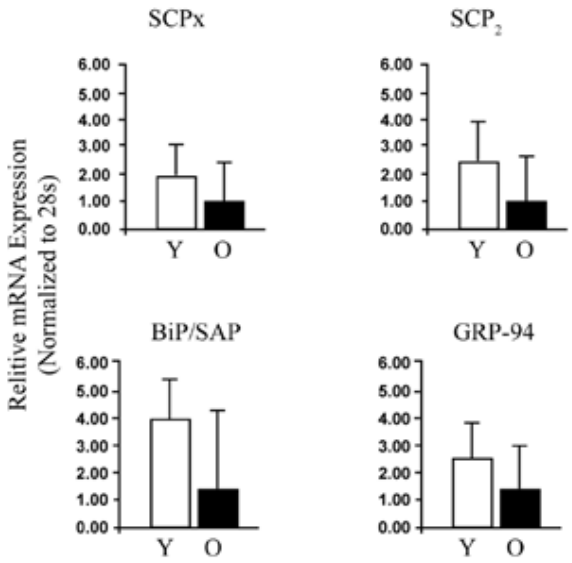

Fig. (4). Real-time quantitative PCR analysis of SCP-x, SCP-2, BiP/GRP-78/SAP and GRP-94 mRNA expression in rat Leydig cells isolated from young (5-Mo, Y) and old (24-27-Mo, O) rats. RNA samples from Percoll purified preparations of Leydig cells from young mature (Y, 5-Mo) and old SD (O, 24-27-Mo) rats were analyzed by real-time quantitative PCR. Results are Mean ± SE of 3 separate experiments.

Effects of Aging on Mitochondrial Cholesterol Content in Leydig Cells

In order to establish a functional correlation between age-induced loss of StarD and PBR/TSPO proteins and impaired steroidogenesis, we measured the cholesterol (steroid precur-

sor) levels in mitochondrial preparations that were isolated from young and old rats and pre-treated with ±Bt_2cAMP (2.5 mM), ±AMG (0.4 mM), or ±CHX (0.1 mM) for 90 min. When CYP11A (P450scc) is inhibited by aminoglutethemide (AMG), cholesterol accumulates into the inner mitochondrial membrane [18,21], whereas cyclohexamide (CHX) inhibition of mitochondrial steroid production is associated with increased accumulation of cholesterol into the outer mitochondrial membrane [19,21]. Under basal conditions, mitochondrial cholesterol content was comparable in Leydig cells from young and old animals (Table **2**). Although Bt_2cAMP treatment caused increased accumulation of cholesterol Leydig cell mitochondrial preparations from both young and old rats, the extent of cholesterol accumulation was much greater in young mitochondria as compared to old mitochondria. Qualitatively, similar results were obtained when aminoglutethimide was replaced with cycloheximide.

DISCUSSION

Considerable evidence now indicates that aging leads to a decline in testosterone levels both in humans and experimental animals. In the present studies we sought to determine the contribution of several putative cholesterol transport proteins in the age-related decline of testosterone production by isolated rat testicular Leydig cells. In agreement with previous observations, the current study demonstrates that freshly isolated and purified preparations of Leydig cells from old rats (24-27-Mo) secrete significantly less testosterone in response to hormone (hCG), a direct stimulator of adenyl cyclase and cAMP production (forskolin), or a cAMP agonist (Bt_2cAMP) as compared to cells derived from the testis of young mature (5-Mo) rats. However, no aging defect was evident, when testosterone production was assessed in response to freely diffusible preparations of cholesterol substrate, suggesting that the age-related decline in testosterone secretion primarily results from the reduced availability of substrate cholesterol at the CYP11A1 sites of the mitochondria. Additionally, a significant correlation was observed between the loss of steroidogenic response, inefficient cholesterol transport to and within the mitochondria and impaired gene expression of key cholesterol transport proteins, StAR/StarD1, StarD2/PC-TP, StarD4 and PBR/TSPO during aging. These observations support the conclusion that aging interferes with Leydig cell testosterone production by restricting the availability of cholesterol substrate in mitochondria through inhibition of the expression of key cholesterol transport proteins crucial for normal steroidogenesis.

Acute testosterone production in Leydig cells, like in other steroid producing cells/tissues, is controlled at the level of cholesterol delivery to the inner membrane, the site of P450 side-chain cleavage (P450scc) enzyme (CYP11A) that converts it to pregnenolone [18-22]. Upon trophic hormone (LH/hCG) stimulation, steroid synthesis in Leydig cells, like in other steroidogenic tissues/cells, is initiated both through the availability of cytosolic free cholesterol [55] and through enhanced cholesterol transfer between outer and inner mitochondrial membranes [56]. This rate limiting process is known to have an absolute requirement for *de novo* synthesis of a labile protein factor [32,57,58]. The well-characterized steroidogenic acute regulatory protein (StAR) fulfills many of the characteristics of this putative labile protein factor [32], i.e., it is synthesized specifically in the adrenal and gonads, is highly labile, and its synthesis is sensitive to the protein synthesis inhibitor, cyclohexamide [32]. The discovery of mutations in the StAR gene in patients with lipoid congenital adrenal hyperplasia was critical to the elucidation of the role of StAR protein in the acute steroidogenic response [59]. Lipoid congenital adrenal hyperplasia patients have markedly impaired gonadal and adrenal steroidogenesis (due to an inability to efficiently transport cholesterol into the mitochondria) associated with massive accumulation of cholesterol in lipid droplets [59]. Deletion of the murine StAR gene by homologous recombination yielded an identical phenotype of impaired steroidogenesis and adrenal and Leydig cell lipid accumulation in StAR null mice [60]. Additional studies further confirmed that StAR functions as a sterol transfer protein [61], binds cholesterol [62,63], mediates cholesterol transfer to the inner mitochondrial membrane [64], and that StAR need not enter mitochondria to produce this transfer [64-66]. StAR is also a prototype of a family of proteins that contain START (StAR-related lipid transfer) domains (StarD proteins) [36], of which StarD3/MLN64, StarD4, StarD5 and StarD6 exhibit steroidogenic potential [38,39].

We observed an age-related marked reduction in the mRNA expression of StAR/StarD1, StarD2 (PC-TP) and StarD4 both under basal conditions and in response to hor-

Table 2. Effects of Bt_2cAMP, Cyclohexamide (CHX), and Aminoglutethimide (AMG) on Mitochondrial Cholesterol Levels in Leydig Cells from Young (5-Mo) and Old (24-27-Mo) Rats*

Additions	Young (5-Mo, Y) Cholesterol (μg/mg mitochondrial protein ±SE)	Old (24-27-Mo, O) Cholesterol (μg/mg mitochondrial protein ±SE)	*P* (**Y** *vs* **O**)
Basal	10.17 ± 1.048	7.73 ± 0.837	NS
Bt_2cAMP (2.5 mM)	15.73 ± 0.865	11.77 ± 0.982	0.0487
AMG (0.4 mM)	15.43 ± 0.865	9.97 ± 0.801	0.0127
CHX (0.1 mM)	13.60 ± 0.851	10.20 ± 0.710	0.0373
Bt_2cAMP + AMG	26.93 ± 2.284	10.00 ± 0.777	0.0022
Bt_2cAMP + CHX	25.70 ± 1.258	10.67 ± 1.648	0.0019

*Results are Mean ±SE of three independent determinations.

monal stimulation using RNA preparations from Leydig cells of old rats (Fig. **2**). However, no age-related changes in the expression of either StarD3/MLN64 or StarD5 were observed. Although StarD3, StarD4 and StarD5 all possess sterol transfer activity and support steroidogenesis to some extent, aging only impacted StAR/StarD1 and StarD4 expression suggesting that these two StarD isoforms play a critical role in the regulation of Leydig cell steroidogenesis. In contrast, aging showed no effect on the other two steroidogenic StarD isoforms, i.e., StarD3/MLN64 and StarD5. The observed lack of an aging affect on StarD3/MLN64 is somewhat surprising given that this protein is most closely related to StAR/StarD1 protein [35,67,68]. It is possible that StarD3/MLN64 (and StarD5) has no or a limited role in promoting steroidogenesis *in vivo*. Indeed, it has been reported recently that targeted mutation of the MLN64 START domain causes only a slight reduction in circulating levels of testosterone in males and gonadotropin-stimulated progesterone levels in females [67]. Finally, at present we have no viable explanation as to how an age-induced reduction in StarD2, a phosphatidylcholine transfer protein (PC-TP, is linked to the decline in steroidogenesis during aging. While PC-TP is a highly specific soluble lipid-binding protein that transfers phosphatidylcholine (PC) between membranes *in vitro*, more recent evidence suggests that it also functions as a PC-sensing molecule that participates in diverse biological functions that are dictated by cellular expression of distinct interacting proteins [69]. Given this, it is possible that StarD2/PC-TP may function as a modulator of the function of certain cholesterol transport proteins or the activities of enzymes involved in testosterone biosynthesis.

Considering the fact that StAR/StarD1 acts on the outer membrane in moving cholesterol from the OMM to the IMM, it raises the possibility that it is a component of a larger molecular complex. Extensive pharmacological studies suggest that the PBR/TSPO is another molecule that facilitates cholesterol transport [28,31]. PBR/TSPO is expressed ubiquitously in the outer mitochondrial membrane, but is most abundant in steroidogenic cells [28,31,39,40]. PBR ligands stimulate steroidogenesis and promote translocation of cholesterol from OMM to the IMM [28,31]. Targeted disruption of the PBR 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 into this cell line restored steroidogenesis [43]. 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 [43,68,70].

It appears now that PBR/TSPO and StAR work in concert to mediate the movement of cholesterol from OMM to IMM. While a physical interaction between StAR and PBR has not been established [71], FRET measurements indicated that StAR and PBR/TSPO come within the 100 A^o of each other [72]. Moreover, Hauet *et al.* [43] 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 [43,71]. Liu *et al.* [73] have provided evidence that hormonal stimulation of a Leydig cell line leads to formation of a StAR-PKARIα-PAP7-TSPO macromolecular signaling complex on the outer mitochondrial membrane that mediates the effect of hormones on mitochondrial cholesterol transport and steroidogenesis. Very recently, evidence was presented to suggest that StAR/StarD1 interacts with VDAC1, and with phosphate carrier protein (PCP) on the OMM to initiate the action of StAR [45]. These various studies point to a functional cooperation between the PBR/TSPO and StAR proteins [35,40,64,68,72] and possibly participation of other StarD proteins as well. Our results demonstrate that aging specifically down-regulates the expression of 18-kDa PBR/TSPO itself. The expression of other components of the PBR/TSPO complex, such as VDAC and ANC, of endogenous ligand, DBI, however, is not impacted by aging (Fig. **3**). Together, our studies suggest that aging drastically affects the expression of both StAR/StarD1 and PBR/TSPO, the two critical proteins that are crucial for optimum cholesterol delivery to the mitochondria and the maintenance of steroidogenesis.

Aside from examining the StarD and PBR/TSPO and its associated proteins, we evaluated the effects of aging on the expression of SCP-2 and the related protein, SCP-x (which are generated from the same gene *via* the use of alternate promoters), and the SAP precursor, BiP/GRP-78. It has been shown that transport of free cholesterol to, and within the mitochondria, may involve SCP-2, which has been identified in various steroidogenic tissues and cells [23,74]. In addition, transfer of cholesterol from OMM to IMM and its association with CYP11A1 may be enhanced by the action of a cycloheximid-sensitive, SAP [25,26]. SAP is an acidic polypeptide, which shares a nearly complete sequence homology with the COOH-terminal region of a minor heat shock protein, 78-kDa glucose-regulated protein, GRP-78 or BiP differing in only two amino acid residues [48,75]. The physiologically active SAP is cleaved from GRP-78/BiP co-translationally in response to a rise in intracellular cAMP levels. Our results suggest that aging has no apparent effect on the mRNA expression of SCP-2, SCP-x, SAP precursor, GRP-78/BiP or another glucose-regulated protein, GRP-94.

In conclusion, we have performed a systematic q-PCR analysis of the impact of aging on the mRNA expression of potential cholesterol transport proteins that may facilitate the delivery of cholesterol to the mitochondrial site of CYP11A1 for side chain cleavage and pregnenolone production (the precursor of all steroids). We provide evidence that aging selectively down-regulates the expression of StAR/StarD1 and PBR/TSPO, the two proteins which are now increasingly recognized as the major players in the cholesterol delivery to and within the mitochondria for steroid production. Beside these two proteins, aging also suppresses the mRNA expression of StarD2/PC-TP and StarD4 although their potential role in cholesterol transport to mitochondria is not yet established. The impaired mRNA expression of StAR/StarD1, PBR/TSPO, StarD2 and StarD4 proteins in the old rat Leydig cells described here suggests that loss of function of these proteins is the principal contributor to the age-related decline in testosterone production. Although the exact mechanism is not well understood, it is quite apparent that aging-induced loss of function of these proteins is responsible for the inefficient delivery of cholesterol into the mitochondria, and as a consequence, diminished conversion of

cholesterol to pregnenolone and reduced testosterone production. This represents a novel cellular mechanism that allows negative modulation of steroidogenesis in response to advancing age. Additional studies are underway in this laboratory to delineate the underlying mechanisms involved in impaired expression of these proteins during aging. A particular focus of these studies is to determine the impact of excessive oxidative stress on the functional expression of these various cholesterol transport proteins. Indeed, previous studies from this laboratory [76] and others [77] have already shown that excessive oxidative stress is central to the age related decline in testosterone production by isolated Leydig cells, and that reactive oxygen species (ROS) can inhibit the expression of StAR/StarD1 in MA-10 mouse Leydig tumor cells [78].

CONCLUSIONS

There is increasing evidence that aging leads to a decline in testosterone levels in both humans and experimental animals. Here we evaluated the effects of aging on the expression of putative cholesterol transport proteins in Leydig cells in relation to age-induced loss of steroidogenic function. Leydig cell testosterone production was significantly decreased in cells isolated from old (24-27-Mo) as compared to young mature (5-Mo) rats. However, no significant aging effect was observed when testosterone production was measured in response to freely-diffusible hydroxycholesterols indicating that age-induced loss of testosterone production primarily results from the inefficient transport of cholesterol substrate to and within the mitochondria. Furthermore, aging significantly diminished the Leydig cell mRNA expression levels of StAR/StarD1, StarD4 and PBR/TSPO. These changes correlated well with reduced accumulation of cholesterol in Leydig cell mitochondria from old animals, especially in response to Bt_2cAMP stimulation and when further metabolism of cholesterol was blocked by the inhibitors of steroidogenesis. Overall, these results suggest that aging inhibits testosterone biosynthesis by diminishing the delivery of cholesterol to and within the mitochondria through impaired expression of the key cholesterol transport crucial for Leydig cell steroidogenesis and consequently decreasing the conversion of cholesterol to pregnenolone and a reduction in testosterone production.

LIST OF ABBREVIATIONS

AMG	=	aminoglutethimide
ANC	=	30-kDa adenine nucleotide translocator
BiP	=	polypeptide binding protein
CHX	=	cycloheximide.
DBI	=	polypeptide diazepam binding inhibitor
endozepin	=	des-(Gly-Ile)-DBI
GRP-78	=	78-kDa glucose-regulated protein
GRP-94	=	94-kDa glucose-regulated protein
hCG	=	human chrionic gonadotropin
IMM	=	inner mitochondrial membrane
OMM	=	outer mitochondrial membrane
P450scc (CYP11A1)	=	cholesterol side-chain cleavage enzyme
PAP7	=	PKA regulatory subunit RIα-associated protein
PBR	=	peripheral-type benzodiazepine receptors
PKA	=	protein kinase A
PRAX-1	=	PBR-associated protein 1
SAP	=	steroidogenesis-activator polypeptide
SCP-2	=	sterol carrier protein-2
SCP-x	=	sterol carrier protein-x
StAR	=	steroidogenic acute regulatory protein
StarD proteins	=	StAR-related lipid transfer (START) domain containing proteins
TSPO	=	translocator protein
VDAC	=	34 kDa voltage-dependent anion channel

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