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Leydig Cell Aging: Steroidogenic Acute Regulatory Protein (StAR) and Cholesterol Side-Chain Cleavage Enzyme

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ABSTRACT: Primary points of control in steroidogenesis are the transport of cholesterol from intracellular stores to the inner mitochondrial membrane, and the subsequent conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme ($P450_{scc}$). Testosterone production has been shown to decline in Brown Norway rat Leydig cells as the rats age. To better understand the mechanism by which aging Leydig cells lose steroidogenic function, we examined the effect of aging on steroidogenic acute regulatory protein (StAR), an important Leydig cell cholesterol transfer protein, and on $P450_{scc}$. Leydig cells isolated from middle-aged (14 months) and old (24 months) rats produced significantly less testosterone than cells from young (4 months) rats. StAR mRNA (1.7 kilobase [kb]) was significantly reduced in Leydig cells from middle-aged and old rats, by 26% and 52%, respectively. Significant reductions also were seen in the steady-state levels of mRNA for $P450_{scc}$, of 29% and 50%, respectively. Western blots revealed significant reductions in StAR protein, by 47% and 74%, respectively, and in $P450_{scc}$ protein, by 38% and 54%, respectively. In response to LH stimulation in vitro, testosterone production by Leydig cells in young, middle-aged, and old rats increased by 30-, 40-, and 33-fold, respectively, although the amounts of testosterone produced by the young cells significantly exceeded that produced by the middle-aged and old cells. StAR protein also increased in response to LH by 1.4-, 3-, and 11-fold, respectively, whereas $P450_{scc}$ protein remained unchanged. These results are consistent with the conclusion that compromise of StAR-mediated cholesterol transport may play a key role in age-related reductions in Leydig cell steroidogenesis. However, because $P450_{scc}$ is reduced in old Leydig cells, the reaction catalyzed by this enzyme would be rate-limiting under circumstances in which saturating amounts of cholesterol entered the mitochondria.

Key words: $P450_{scc}$, testosterone, testis, rat.

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During aging, the capacity of Leydig cells isolated from the testes of Brown Norway rats to produce testosterone in response to luteinizing hormone (LH) stimulation significantly declines with age (Chen et al, 1994, 1996). This decline in steroidogenic capacity does not appear to be due to reduced LH levels (Chen et al, 1994, 1996; Gruenewald et al, 1994; Bonavera et al, 1997, 1998; Grzywacz et al. 1998), but rather to age-related changes in the Leydig cells themselves. In this regard, in previous studies we reported that there are reductions in the activities of each of the steroidogenic enzymes responsible for converting cholesterol to testosterone, including P450 cholesterol side-chain cleavage ($P450_{scc}$) enzyme, Δ^5-3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD), 17α-hydroxylase/C_{17-20} lyase ($P450_{17\alpha}$), and 17β-hydroxysteroid dehydrogenase (17β-HSD; Luo et al, 1996). Reductions in the activity of any of these enzymes could cause reduced steroidogenic function in aging Leydig cells.

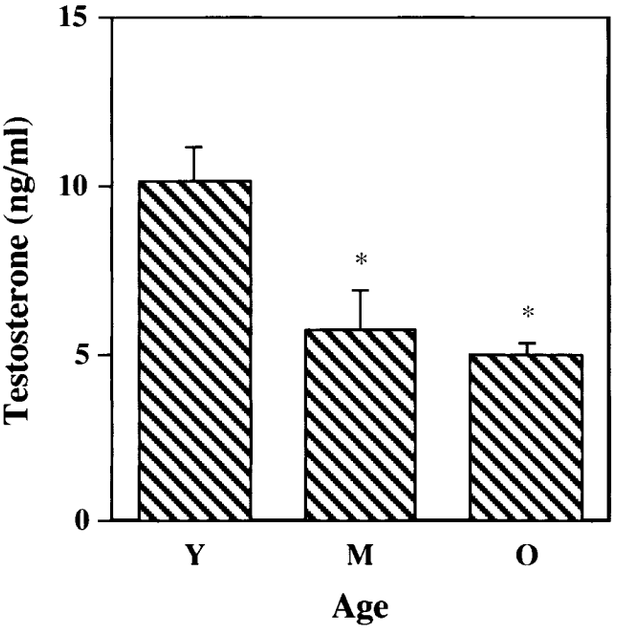

Figure 1. Basal testosterone production by Leydig cells isolated from young (Y, 4-mo-old), middle-aged (M, 14-mo-old), and old (O, 24-mo-old) rat testes. Cells from M and O rats produced significantly less testosterone than cells from young rats. *Significantly different from Y value ($P < .05$).

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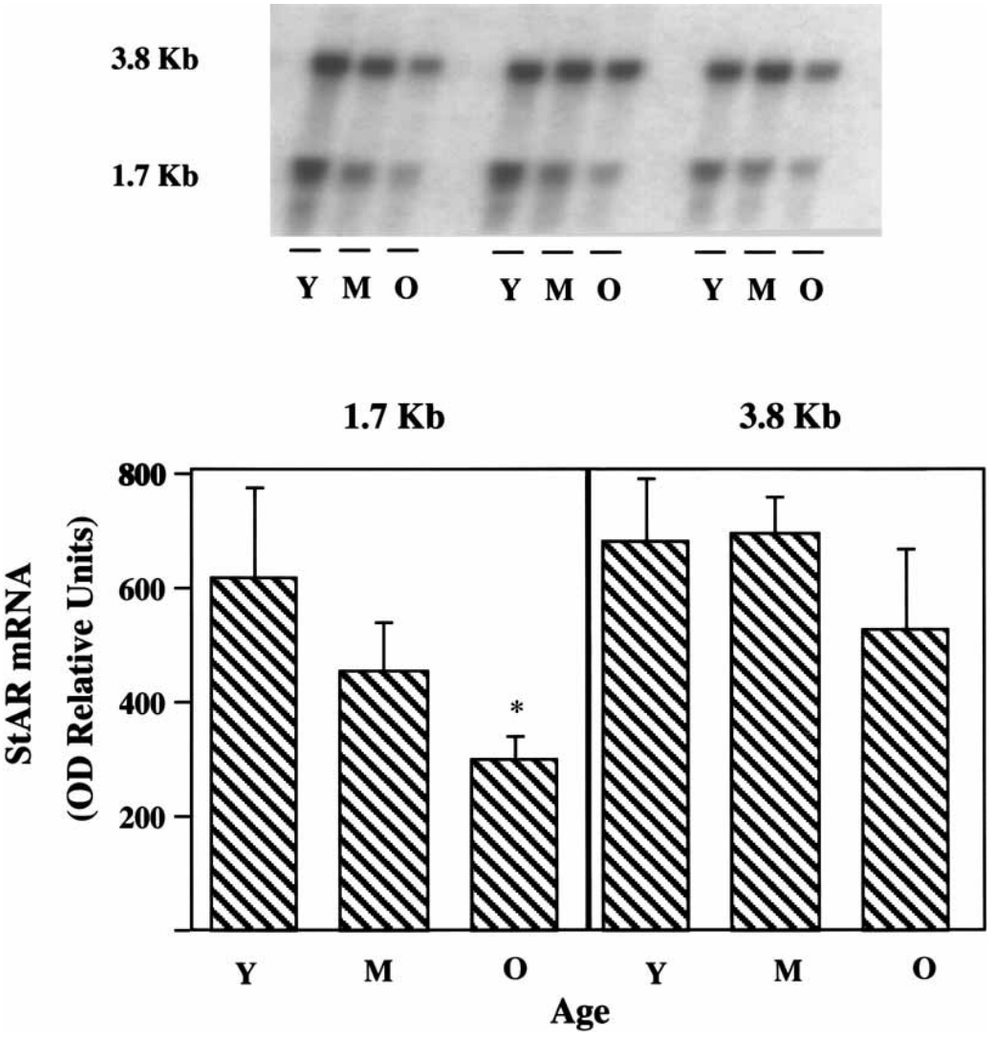

Figure 2. **Above**: Representative Northern blots of Leydig cell StAR mRNA transcripts (3 separate experiments). Cells were isolated from young (Y), middle-aged (M), and old (O) rat testes. **Below**: Quantitative analysis of the Northern blots. In comparison to the 1.7-kb transcript in young cells, there was a 26% decrease in this transcript in cells from M rats, and a significant (52%) decrease in cells from O rats. No age-related changes were seen in the 3.8-kb transcript. *Significantly different from Y value ($P < .05$).

It is well established that the primary points of control in the acute stimulation of steroidogenesis by LH are the transport of cholesterol from intracellular stores to the inner mitochondrial membrane (Crivello et al, 1980; Privalle et al, 1983; Stocco, 1996, 1999), the site of $P450_{scc}$ (Farkash et al, 1986), and the subsequent conversion of cholesterol to pregnenolone by this enzyme (Saez, 1994). With this in mind, our objective was to determine whether the reduced testosterone production that characterizes old Leydig cells may be explained by age-related changes in an important Leydig cell mitochondrial cholesterol transfer protein, steroidogenic acute regulatory protein (StAR; Clark et al, 1994; Luo et al, 1998; Stocco 1999), or in $P450_{scc}$, or both. We show that StAR protein and steady-state mRNA are reduced in old Leydig cells, as are $P450_{scc}$ activity, protein, and steady-state mRNA. In response to LH in vitro, there was a rapid increase in testosterone production in Leydig cells from young, middle-aged, and old rats that was correlated with increases in StAR protein but not in $P450_{scc}$ protein. These results are consistent with the conclusion that compromise of StAR-mediated cholesterol transport may be of critical importance in age-related reductions in Leydig cell steroidogenesis. However, because $P450_{scc}$ is reduced in old Leydig cells, the reaction catalyzed by this enzyme would be rate-limiting in circumstances in which saturating amounts of cholesterol entered the mitochondria, whether or not StAR was reduced.

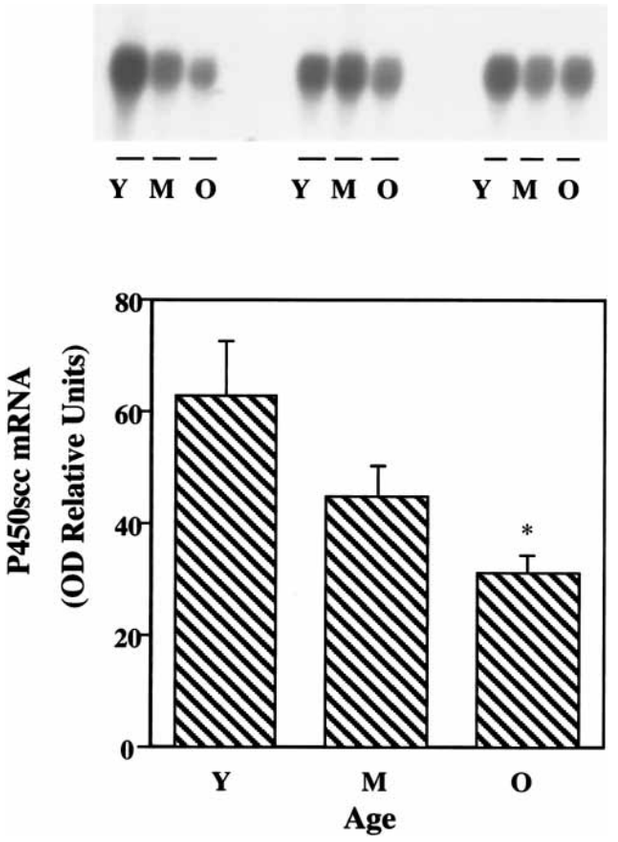

Figure 3. **Above**: Northern blots of Leydig cell $P450_{scc}$ mRNA transcripts. Cells were isolated from young (Y), middle-aged (M), and old (O) rat testes. **Below**: Quantitative analysis of the Northern blots. $P450_{scc}$ was reduced in cells from M (29%) and O (50%) rats. *Significantly different from Y value ($P < .05$).

Materials and Methods

Animals

Adult male Brown Norway rats aged 4 (young), 14 (middle-aged), and 24 (old) months were used in these studies. Rats were obtained through the National Institute on Aging, supplied by Harlan Sprague-Dawley, Inc (Indianapolis, Ind). They were housed in controlled light (14 hours of light and 10 hours of dark) and temperature (22°C) conditions with free access to rat chow (Prolab RMH 1000, PMI Nutrition International, Inc, Brentwood, Mo) and water. All procedures were in accord with protocols approved by The Johns Hopkins University Animal Care and Use Committee.

Isolation and Culture of Leydig Cells

Leydig cells were isolated and purified by centrifugal elutriation and Percoll density gradient centrifugation as previously described (Klinefelter et al, 1987). The purity of the Leydig cell preparations was consistently greater than 93% as assessed by determining the percentage of cells that stained histochemically for 3β-HSD (Klinefelter et al, 1987). At each age of the 3 ages under study, Leydig cells were purified from 4 groups of 2–3 rats each (4–6 testes) and pooled. Leydig cells (1.5×10^5) were placed in tissue culture dishes without or with maximally stimulating ovine LH (100 ng/mL, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md) for 4 hours. Culture media were then centrifuged and the supernatants were saved for subsequent testosterone assay. Cells were suspended in sample buffer for Northern and Western blot analyses.

Testosterone Radioimmunoassay

Testosterone concentrations were determined in 10-μL aliquots of medium by radioimmunoassay (RIA) according to methods described previously (Schanbacher et al, 1975). The assay sensitivity was 10 pg/sample, with intra-assay and interassay coefficients of variation of 11.2% and 9.6%, respectively.

Northern Blot Analysis

Total RNA was extracted from freshly isolated Leydig cells by a single-step method described previously (Chomczynski and Sacchi, 1987; Kedzierski and Porter, 1991) with modifications. Prior to RNA extraction, ^{35}S-labeled RNA (10 000 cpm) was added to each sample in order to determine the percent recovery of total RNA (Wright et al, 1993). Total RNA from equal numbers of Leydig cells was analyzed.

Complementary DNA (cDNA) was labeled with deoxycytidine 5′-[α-^{32} P] triphosphate (dCTP) to approximately 1×10^8 cpm/μg DNA using a random primer synthesis method (Megaprime DNA Labeling System, Amersham, Piscataway, NJ). Northern blot analysis was carried out as described previously (Sambrook et al, 1989). Total RNA from 1×10^6 cells was loaded per lane and electrophoresed through a denaturing 1.2% agarose gel. The gels were subsequently blotted by capillary action to a nylon filter. An RNA ladder, 0.24–9.5 kilobase (kb; Gibco BRL, Life Technologies, Inc, Gaithersburg, Md), which was visualized by ethidium bromide staining, was used as a molecular size marker. The following cDNA clones were used: 1.5-kb mouse StAR cDNA (Clark et al, 1994) and 1.2-kb rat $P450_{scc}$ cDNA (Oonk et al, 1989). Northern blots were hybridized with the appropriate ^{32}P-labeled cDNA probes (1×10^6 cpm/mL) for a minimum of 16 hours. Excess label was removed by 2 washes at room temperature in 2× SSC (0.15 M NaCI, 0.15 M sodium citrate pH 7.0) − 0.1% sodium dodecyl sulfate (SDS) for 20 minutes, and then once at 60°C in 0.1× SSC-0.5% SDS for 40 minutes. Nylon membranes were sequentially probed for StAR, $P450_{scc}$, and 18S rRNA. Blots were exposed to x-ray film at −70°C with an intensifying screen. The integrated optical density (OD) of the hybridized probes was determined from autoradiographs of representative Northern blots by National Institutes of Health (NIH) image analysis software (Bethesda, Md). The ODs were determined from several autoradiographs of each blot; exposures within the linear range of the film were used to quantify the probes. The OD values for StAR and $P450_{scc}$ were normalized to the OD value of 18S rRNA.

Immunoblot Analysis

Purified Leydig cells from young, middle-aged, and old rats were incubated without or with LH for 4 hours in vitro as described above and previously (Chen et al, 1994). Whole-cell homoge-

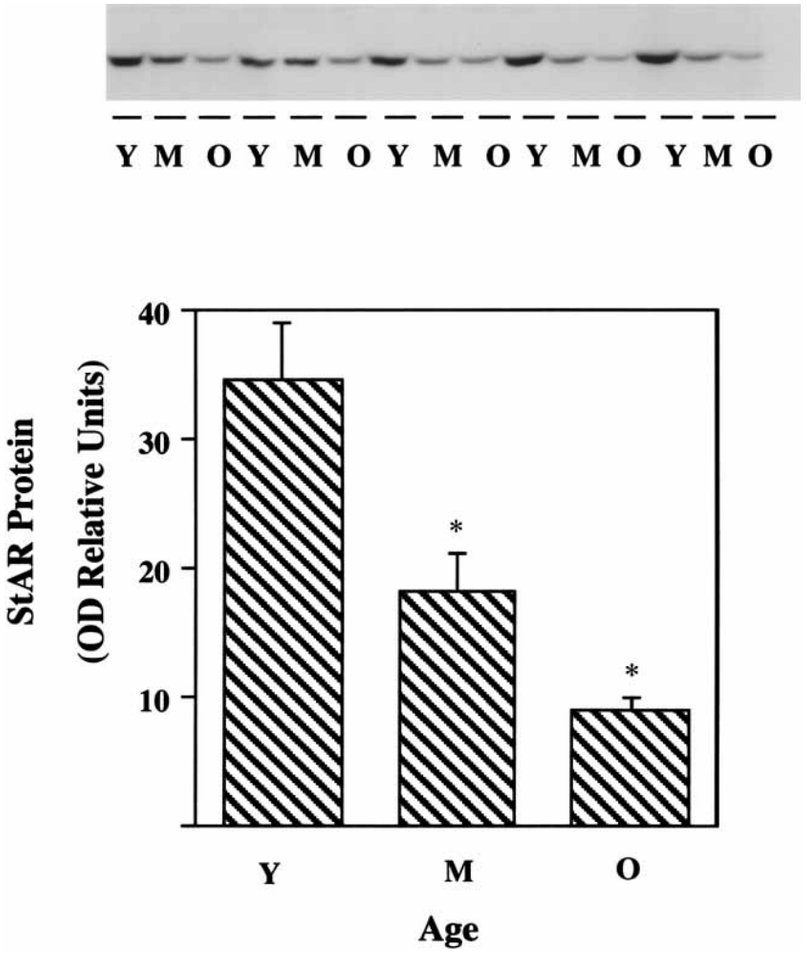

Figure 4. **Above**: Western blots of Leydig cell constitutive StAR protein (5 separate experiments). Cells were isolated from young (Y), middle-aged (M), and old (O) rat testes. **Below**: Quantitative analysis of the Western blots. The 30-kd StAR (mature form) protein content was reduced in cells from M (47%) and O (74%) rats. *Significantly different from Y value ($P < .05$).

nates were solubilized in sample buffer (50 mM Tris pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, and 0.001% bromophenol blue). Proteins were separated by 12% polyacrylamide gel electrophoresis with each lane containing protein from equal numbers of cells (2×10^5), and then electrotransferred onto a nitrocellulose filter. The rabbit antibodies with which blots were incubated were StAR 1:1000 (Clark et al, 1994) and $P450_{scc}$ 1:10 000 (Chemicon International Inc, Temecula, Calif). Subsequently, blots were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; donkey IgG, Amersham Pharmacia Biotech, Piscataway, NJ), and an enhanced chemiluminescence kit was used to detect the horseradish-peroxidase-labeled protein according to the manufacturer's instructions (Amersham Pharmacia Biotech). The x-ray films were quantified by densitometry.

Statistical Analyses

Data are expressed as the mean ± standard error of the mean (SEM). As indicated above, Leydig cells were obtained from the testes of 4 groups of 2–3 rats at each age studied. The means and SEMs were based on the 4 groups of pooled cells. Statistical differences involving multiple group comparisons were determined by one-way analysis of variance (ANOVA). If group differences were revealed by ANOVA ($P < .05$), differences between individual groups were determined with the Scheffé F-test ($P < .05$).

Results

Age-Dependent Changes in Leydig Cell Testosterone Production

Figure 1 shows basal (nonstimulated) testosterone production by Leydig cells from young, middle-aged, and old rats. The cells were incubated for 4 hours in the absence of LH stimulation. There was an age-dependent reduction in testosterone production; Leydig cells from middle-aged and old rats produced significantly less testosterone than did cells from young rats.

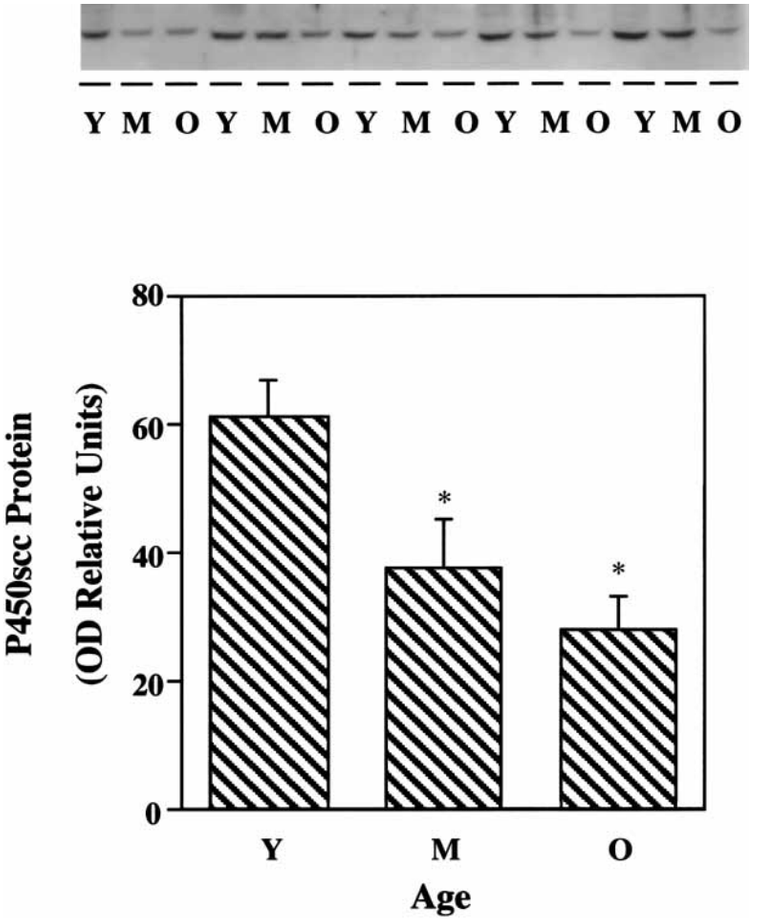

Figure 5. **Above**: Western blots of Leydig cell $P450_{scc}$ protein. Cells were isolated from young (Y), middle-aged (M), and old (O) rat testes. **Below**: Quantitative analysis of the Western blots. $P450_{scc}$ was reduced in cells from the M (38%) and O (54%) rats. *Significantly different from Y value ($P < .05$).

Age-Dependent Changes in Leydig Cell StAR and $P450_{scc}$ mRNA and Protein Contents

Figure 2 shows Northern blots of StAR mRNA transcripts derived from 3 separate experiments and quantitative analyses of the blots. The lanes were loaded with mRNA from equal numbers of cells from young, middle-aged, and old rats. Three StAR transcripts were seen, two of which (1.7 and 3.8 kb) were prominent, whereas the third (1.2 kb, not evident) was barely detectable. In comparison with the transcripts in Leydig cells from young rats, there was a 26% decrease in the quantity of the 1.7-kb transcript in cells from the middle-aged rats and a significant (52%) decrease in this transcript in cells from old rats. No significant age-related changes were seen in the 3.8-kb StAR mRNA.

Figure 3 shows Northern blots of $P450_{scc}$ mRNA transcripts and quantitative analyses of these blots. As is evident, aging reduces $P450_{scc}$; reductions from the young value of 29% and 50% (significant), were seen in cells from middle-aged and old rats, respectively. Figure 4 shows Western blots of constitutive StAR proteins from 5 separate experiments and quantitative analyses of the levels of the blots. Lanes were loaded with protein from equal numbers of cells and then quantified by scanning densitometry. The 30-kd StAR (mature form) protein content was significantly reduced with age, by 47% and 73% in cells from middle-aged and old rats, respectively. Figure 5 shows Western blots of $P450_{scc}$ protein along with quantitative analyses of the blots. $P450_{scc}$ also was significantly reduced, by 38% and 54% in middle-aged and old rat Leydig cells, respectively. Similar results were obtained when equivalent amounts of protein were loaded onto the gels (not shown).

Effects of LH and Age on Testosterone Production and on StAR and $P450_{scc}$ Proteins

Freshly isolated Leydig cells from young, middle-aged, and old rats were incubated for 4 hours in the absence or

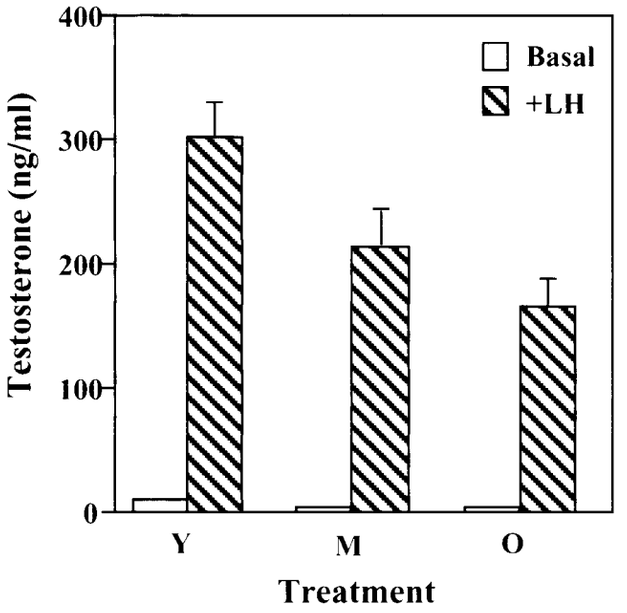

Figure 6. Testosterone production by Leydig cells isolated from young (Y), middle-aged (M), and old (O) rat testes. The cells were incubated without LH (basal) or with maximally stimulating LH. LH stimulation resulted in increases of 30-, 40-, and 33-fold in the ability of cells from Y, M, and O rats, respectively, to produce testosterone.

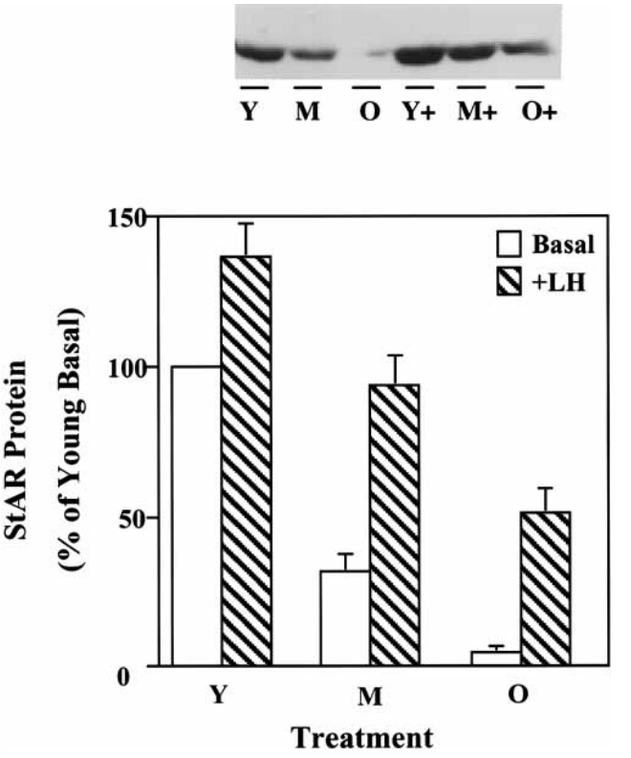

Figure 7. **Above**: Western blots of Leydig cell StAR protein. Cells were isolated from young (Y), middle-aged (M), and old (O) rat testes, and incubated without LH (basal) or with maximally stimulating LH (+). **Below**: Quantitative analysis of StAR protein. Incubation of Leydig cells with LH resulted in increases in the 30-kd StAR protein at all ages.

presence of maximally stimulating ovine LH. Testosterone production by these cells was assayed, or the cells were prepared for Western blot analysis of StAR and $P450_{scc}$. Figure 6 shows that LH stimulation resulted in comparable increases of 30-, 40-, and 33-fold in the ability of Leydig cells from young, middle-aged, and old rats, respectively, to produce testosterone, although the amounts of testosterone produced by the young cells was significantly greater than by the middle-aged or old cells. Quantitative analyses of Western blots derived from 5 separate experiments (one of which is shown in Figure 7, top) revealed that incubation of Leydig cells from young, middle-aged, and old rats with LH resulted in increases in the 30-kd StAR protein, in all cases, of 1.4-, 3-, and 11-fold, respectively (Figure 7). In striking contrast, LH stimulation did not result in increased $P450_{scc}$ protein over basal levels in Leydig cells from any age (Figure 8).

Discussion

It is now well established that a critical point of control in the acute stimulation of steroidogenesis by LH is the transport of cholesterol from intracellular stores to the inner mitochondrial membrane, and thus to the first enzyme of the steroidogenic cascade, $P450_{scc}$, which converts cholesterol to pregnenolone (see review by Stocco, 1999). The $P450_{scc}$-catalyzed step, the first reaction in steroidogenesis in all steroidogenic tissues (Stocco, 1999), can be rate-limiting if cholesterol enters the mitochondria in large quantity. However, the rapid increased steroidogenesis that results from the acute stimulation of steroidogenic cells with trophic hormone typically is not related to the activity of $P450_{scc}$, but rather to the movement of cholesterol into the mitochondria (Crivello et al, 1980; Privalle et al, 1983). For example, steroidogenic cells have been shown to produce a maximum amount of steroid even in the absence of hormonal stimulation when the cells are incubated with hydrophilic, cholesterol-like substrates that diffuse to the inner mitochondrial membrane (Chaudhary and Stocco, 1988). This indicates that, depending upon how much cholesterol is available for cleavage within the mitochondria, pregnenolone production may be increased or decreased without a change in $P450_{scc}$.

How does this relate to aging Leydig cells? The results presented herein show that StAR protein is significantly diminished—by 74%—in old Leydig cells. This study and a previous study showing a decrease in StAR protein in aging Leydig cells (Leers-Sucheta et al, 1999) suggest the possibility that there may be reduced cholesterol transfer to the $P450_{scc}$ enzyme within the mitochondria of

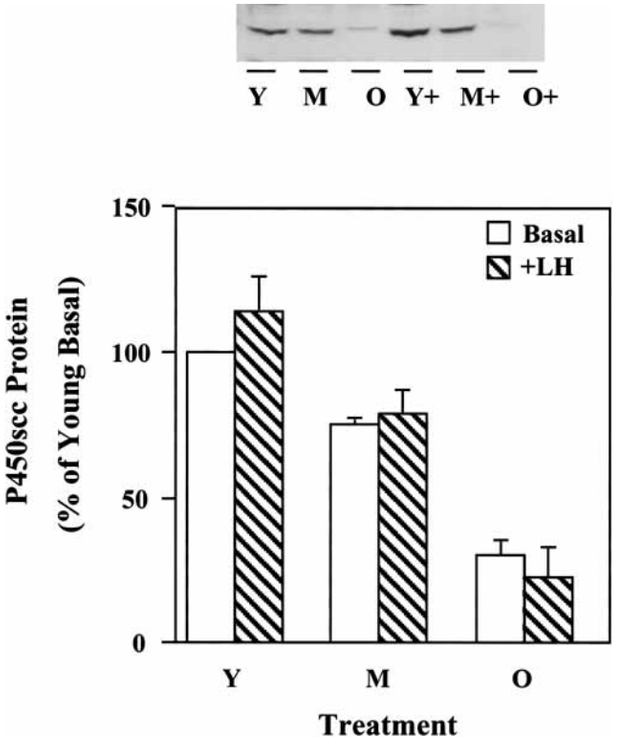

Figure 8. **Above**: Western blots of Leydig cell $P450_{scc}$ protein. Cells were isolated from young (Y), middle-aged (M), and old (O) Leydig cells, and incubated without LH (basal) or with maximally stimulating LH (+). **Below**: Quantitative analysis of $P450_{scc}$ protein. In striking contrast to StAR, LH stimulation did not result in increased $P450_{scc}$ protein over basal levels in Leydig cells from any age.

old Leydig cells, and therefore, compromise of StAR may play a critical role in the mechanism by which reductions occur in Leydig cell steroidogenesis with aging. However, although this is an attractive possibility, there are others. For example, $P450_{scc}$ was found in this study to be reduced in old cells, and previous studies (Luo et al, 1996) have shown that age-related reductions occur in each of the steroidogenic enzymes downstream from $P450_{scc}$, including 3β-HSD, $P450_{17\alpha}$, and 17β-HSD. Compromise of any one of these steroidogenic enzymes could account for the reduced testosterone production of old Leydig cells if the compromise resulted in nonsaturating substrate being presented to the succeeding enzymatic step. At this juncture, we do not know which of the reactions of the steroidogenic pathway is rate-limiting, or perhaps more importantly, the mechanism or mechanisms by which changes occur in any of the reactions.

Most steroidogenic cells have a basal level of steroid production that is rapidly increased by hormonal stimulation. Leydig cells were found to be no exception; in both young and old cells, LH stimulation in vitro produced a 30-fold to 40-fold increase in testosterone production in only 4 hours, although the young cells produced far more testosterone than the old cells did. The observation of equivalent fold-increases in testosterone production by young and old cells provided an experimental means by which to examine the possibility of a role for StAR in the reduced steroidogenesis of old cells. As indicated earlier, the basal level of StAR protein was found to be significantly (74%) lower in old cells than in young cells, suggesting that if the stores of cholesterol within young and old cells were equivalent, StAR-related reductions in cholesterol movement to the inner mitochondrial membrane may account, at least in part, for age-related reductions in testosterone production. When Leydig cells from young and old rats were incubated with maximally stimulating LH for 4 hours, there was a rapid increase in StAR in both young and old cells, with the fold-increase far greater in the old (11-fold) than the young (1.4-fold) cells. During the same 4-hour period of treatment, there was no change in $P450_{scc}$ in either young or old cells. Correlation between the increases in testosterone production and StAR in response to LH suggests that the testosterone increases may result from increased cholesterol transport to the $P450_{scc}$ enzyme of the inner mitochondrial membrane. This possibility is consistent with a number of studies of StAR's involvement in steroidogenesis. For example, important evidence for the role of StAR in steroidogenesis has come from studies of patients with congenital lipoid adrenal hyperplasia (Lin et al, 1995), a disorder characterized by an inability to convert cholesterol to pregnenolone. In individuals with this disorder, StAR was shown to be mutated, but $P450_{scc}$ to be normal, supporting the contention that StAR mediates hormone-induced steroid hormone synthesis. In addition, in a recent study in which the relationship between protein synthesis and testosterone production by rat Leydig cells in primary culture was examined, a temporal relationship between synthesis of the StAR proteins and the stimulation of testosterone production was shown (Luo et al, 1998), providing correlative evidence for a role for StAR protein in the acute regulation of Leydig cell testosterone biosynthesis by LH.

The results of the present study provide further correlative evidence for the involvement of StAR in the age-related changes in the Leydig cell testosterone production. However, the studies do not rule out the possibility that other cholesterol transport molecules, notably peripheral benzodiazepine receptor (PBR; Papadopoulos, 1993; Boujrad et al, 1994), may play important, indeed perhaps more important, roles in Leydig cell aging. Also not ruled out is the possibility that cholesterol stores within young and old Leydig cells are not equivalent. If the cholesterol stores of old cells were reduced from those of young cells, for example, a consequence may be less cholesterol transport into the mitochondria even if cholesterol transport mechanisms were operating as they do in young cells. By

this scenario, the most profound problem with old cells may be reduced cholesterol content because this, if it pertained, could result in reduced substrate at steps along the steroidogenic pathway. This possibility is currently under study in our laboratory.

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