

Temporal Relationships Among Testosterone Production, Steroidogenic Acute Regulatory Protein (StAR), and P450 Side-Chain Cleavage Enzyme (P450scc) During Leydig Cell Aging

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ABSTRACT: Previous studies have shown that the capacity of Leydig cells from aged (21–24-month-old) Brown Norway rats to produce testosterone is reduced from young (4-month-old) levels, and that this is correlated with reductions in steroidogenic acute regulatory protein (StAR), peripheral benzodiazapine receptor (PBR), and the levels and activities of the steroidogenic enzymes. The age(s) at which particular changes in the steroidogenic pathway occur, and the relationship of particular changes to reduced testosterone production, are not known. We examined 3 critical components of the steroidogenic pathway, cyclic adenosine monophosphate (cAMP) production, StAR, and P450 side-chain cleavage enzyme (P450scc) in relationship to age-related decreases in testosterone production. Leydig cells isolated from Brown Norway rats of increasing ages (4, 9, 15, and 20 months) were evaluated. The ability of Leydig cells to produce testosterone was reduced at 9 months, although not significantly. Significant reductions in testosterone production were first

seen in cells isolated from rats of 15 months of age, and further reductions occurred thereafter. Reduced testosterone was correlated with reductions in StAR, P450scc mRNA, and protein. Significant decline in luteinizing hormone–stimulated intracellular cAMP levels was seen by 9 months, before significant reductions in testosterone, StAR, and P450scc. Further declines in cAMP levels were seen at 15 and 20 months. These studies suggest that age-related reductions in intracellular cAMP may lead to the reduced testosterone production that characterizes aged Leydig cells. This suggestion is supported by recent studies from our lab demonstrating that long-term (3 days) culture of old Leydig cells with dbcAMP restored testosterone production to levels approximating those of young cells.

Key words: Leydig cell, cyclic AMP, steroidogenesis, Brown Norway rat.

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With aging, the capacity of the testes of Brown Norway rats, or of Leydig cells isolated from the testes of these rats, to produce testosterone in response to maximally stimulating luteinizing hormone (LH) declines significantly (Chen et al, 1994, 1996). Previous studies have reported that reduced steroidogenesis is associated with reductions in cyclic adenosine monophosphate (cAMP); the cholesterol transport proteins, steroidogenic acute regulatory protein (StAR) and peripheral benzodiazapine receptor (PBR); and the activities of each of the enzymes involved in steroidogenesis, including the P450 side-chain cleavage enzyme (P450scc), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), P450c17, and 17-ketosteroid reductase (Luo et al, 1996, 2001; Culty et al, 2002). Northern and Western blot analyses revealed that age-related changes

in P450scc, 3 β -HSD, and P450c17 were consistent with enzyme activity analyses (Luo et al, 1996). These observations suggest that the reduced ability of aging Leydig cells to convert cholesterol to testosterone may be a consequence of deficits at multiple points along the steroidogenic pathway, including mitochondrial import of cholesterol (Leers-Sucheta et al, 1999) and the subsequent metabolism of cholesterol to testosterone by enzymes of the mitochondria and smooth endoplasmic reticulum (Ewing and Zirkin, 1983).

As yet, we do not know which among the Leydig cell deficits that accompany Leydig cell aging in fact is *responsible* for reduced steroidogenesis. It seems unlikely that each of the deficits associated with Leydig cell aging occur independently of one another, but rather that an initiating event occurs that leads to coordinate downstream changes in cholesterol import and/or the steroidogenic machinery.

It has long been known that cyclic AMP, produced in response to LH, phosphorylates a number of proteins and, in doing so, directly or indirectly causes increased synthesis of androgen (Stocco, 1992; Clark and Stocco,

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1996). Under normal circumstances, the rate-limiting step of steroidogenesis is cholesterol transport to the inner mitochondrial membrane, a step in which StAR (Stocco, 1996) and PBR (Culty et al, 2002; Liu et al, 2003) play important roles. This is followed by the important first step of converting cholesterol to pregnenolone by the mitochondrial P450_{scc}. Herein we examine cAMP production, StAR, and P450_{scc} in relationship to testosterone production by Leydig cells isolated from the testes of Brown Norway rats of increasing ages (4, 9, 15, and 20 months). The major objectives of the study were to determine the age(s) at which changes in cAMP, StAR, and P450_{scc} occur, and the temporal relationship of such changes to age-related reductions in Leydig cell testosterone production.

Materials and Methods

Animals

Adult male Brown Norway rats 4, 9, 15, and 20 months of age were obtained through the National Institute on Aging, supplied by Harlan Sprague Dawley Inc (Indianapolis, Ind). A total of 150 rats were used for these studies; the numbers per study are presented in the figure legends. The rats were housed under controlled light (14:10 hours light:dark) and temperature (22°C), with free access to rat chow 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 in this study consistently was greater than 93%, as assessed by determining the percentage of cells that stained histochemically for 3 β -HSD (Klinefelter et al, 1987). Leydig cells (2×10^5) 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 Kidney Disease), dibutyryl cyclic adenosine monophosphate (1 mM), 22R-hydroxycholesterol (20 μ M), or pregnenolone (2.5 μ M). All treatments were performed at 34°C for 2 hours.

Testosterone Radioimmunoassay (RIA)

Testosterone concentrations were determined in aliquots of culture medium or serum by RIA, according to methods described previously (Schanbacher and Ewing, 1975). The assay sensitivity was 10 pg/tube, with intra-assay and interassay coefficients of variation of 11.2% and 9.6%, respectively.

cAMP Production

Leydig cells were incubated with maximally stimulating LH (100 ng/ml) for 2.5, 5, 10, 15, or 20 minutes. Immediately afterward, cells were extracted and cAMP was assayed with a cAMP [³H] assay system (Amersham Pharmacia Biotech, Pis-

cataway, NJ), according to the manufacturer's directions. The sensitivity of the assay was 0.05 pmol per assay tube.

Isolation of RNA and Northern Blot Analysis

Total RNA was extracted from freshly isolated Leydig cells by a single-step method (Chomczynski and Sacchi, 1987; Kedzierski and Porter, 1991). Prior to RNA extraction, ³⁵S-labeled RNA (10000 counts per minute [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.

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 subsequently were blotted by capillary action to a nylon filter and were further hybridized with a ³²P-labeled complementary DNA (cDNA) probe for a 1.5 Kb mouse StAR cDNA (Clark et al, 1994) and a 1.2-Kb rat P450_{scc} cDNA (Oonk et al, 1989). cDNA was labeled with [α -³²P] deoxycytidine triphosphate to approximately 1×10^8 cpm/ μ g DNA, using a random primer synthesis method (Megaprime DNA labeling system, Amersham, Piscataway, NJ). Nylon membranes were probed sequentially for StAR, P450_{scc}, and 18S rRNA.

Immunoblot Analysis

Purified Leydig cells from 4-, 9-, 15-, and 20-month-old rats were solubilized in sample buffer (50 mM Tris, pH 6.8, 5% β -mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, and 0.001% bromophenol blue). Proteins were separated by 10% polyacrylamide gel electrophoresis, with each lane containing protein from equal numbers of cells (2×10^5), and then electrotransferred onto a nitrocellulose filter. Blots were incubated with rabbit antibodies to StAR (Clark et al, 1994) and P450_{scc} (Chemicon International Inc, Temecula, Calif). Subsequently, blots were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (donkey IgG, Amersham Pharmacia Biotech, Piscataway, NJ), and an enhanced chemiluminescence (ECL) kit was used to detect the horseradish-peroxidase-labeled protein, according to the manufacturer's instructions (Amersham). The x-ray films were quantified by densitometry.

Statistical Analyses

Data are expressed as the mean \pm standard error of the mean. Statistical differences involving multiple group comparisons were determined by 1-way analysis of variance (ANOVA). If group differences were revealed by ANOVA ($P < .05$), differences between individual groups were determined with the Scheffe F test ($P < .05$).

Results

Figure 1 shows testosterone production by Leydig cells isolated from the testes of 4-, 9-, 15-, and 20-month-old rats and cultured for 2 hours in the absence (basal) or presence of maximally stimulating LH (100 ng/ml). LH

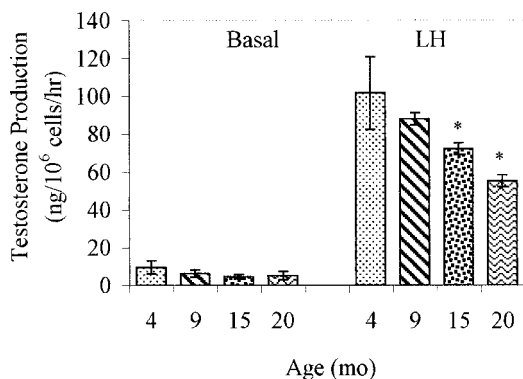


Figure 1. Testosterone production by Leydig cells isolated from rats of 4, 9, 15, and 20 months of age. For each age, Leydig cells were isolated, and pooled, from the testes of 6 groups of 2–3 rats. Each set of pooled cells was plated in 3 replicate wells and incubated without luteinizing hormone (LH) (basal) or with maximally stimulating LH (100 ng/ml). Testosterone concentrations were determined for each of the wells, and the mean determined. The grand mean and standard error of the mean were determined from the 6 values that were generated per age. *Significant difference from the 4-month-old value ($P < .001$).

stimulation resulted in significant increases in testosterone production from basal levels by cells of each age. In response to LH, cells from the 9-month-old rats produced somewhat less testosterone than cells from 4-month-old rats; the difference was not significant. At 15 and 20 months, significant reductions from the young (4 months) production level were seen. Reductions in testosterone levels in the serum of the 4–20-month-old rats were consistent with the decreased capacity of the Leydig cells to produce testosterone (not shown).

With Leydig cells from each of 4-, 9-, 15-, and 20-month-old rats, peak intracellular cAMP levels were reached by 5–10 minutes of incubation with LH (Figure 2A). Age-related decreases in cAMP production were seen; cAMP production, greatest in cells from 4-month-old rats, was reduced by 9 months, and reduced further in cells from 15- and 20-month-old rats. At 20 minutes of incubation with LH, cells from 20-month-old rats produced about half the cAMP of cells from 4-month-old rats.

Figure 2B shows testosterone production by the same Leydig cells that were assessed for cAMP production, in this case from rats of 4–15 months of age. As expected, age-related decreases were seen in the ability of the Leydig cells to produce testosterone. In striking contrast to cAMP production, which peaked at 5–10 minutes of incubation with LH, testosterone production by these cells was still rising at the end of a 20-minute incubation period.

StAR protein is involved in the movement of cholesterol to the inner mitochondrial membrane, the step in the steroidogenic pathway that is considered to be rate limiting. We examined the effect of aging on StAR as well as on P450scc, the mitochondrial enzyme responsible for

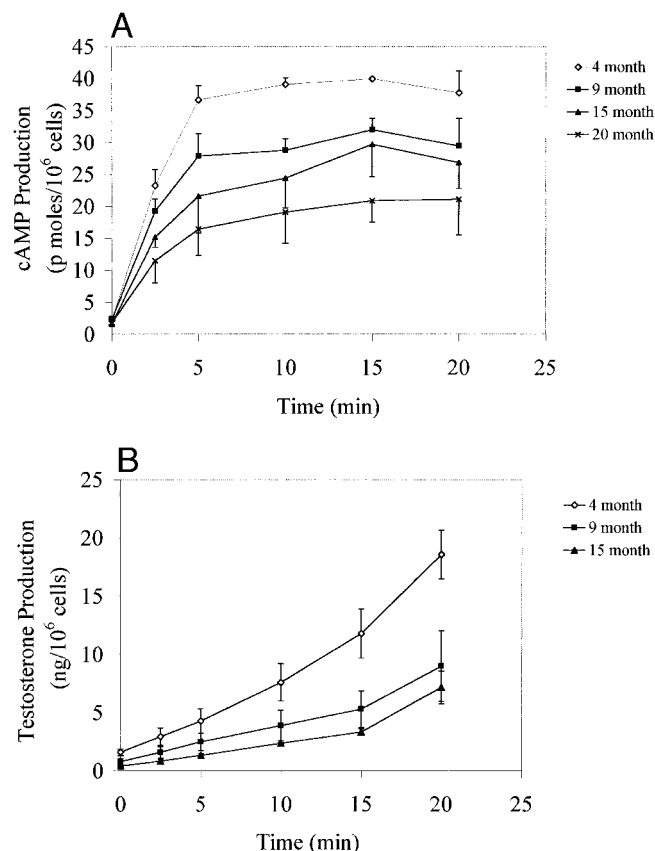


Figure 2. Effect of aging on cyclic adenosine monophosphate (A) and testosterone (B) production. Leydig cells from 4-, 9-, 15-, and 20-month-old rats were cultured with 100 ng/ml of luteinizing hormone for 0–20 minutes. The cells were extracted immediately after incubation. Intracellular cAMP and testosterone in the medium were measured by radioimmunoassay. The bars are the mean \pm standard error of the mean, generated as described for Figure 1.

converting cholesterol to pregnenolone. Leydig cells were isolated from 4-, 9-, 15-, and 20-month-old rats and assessed for steady-state mRNA (Figure 3) and immunoreactive protein (Figure 4) for StAR and P450scc. Northern blot analysis revealed that StAR mRNA was expressed as 2 major transcripts, of 3.8 Kb and 1.7 Kb, and 1 minor transcript, of 1.2 Kb. The 1.7 Kb transcript was reduced in Leydig cells from testes of 9-, 15-, and 20-month-old rats compared to cells from 4-month-old rats (Figure 3), although not significantly at 9 months. P450scc mRNA also was reduced at each age, although not significantly until 15–20 months. Western blot analysis (Figure 4 above and below) similarly revealed decreases in StAR and P450scc proteins; StAR was reduced by 60% from the young value in Leydig cells from 15-month-old rats, and P450scc protein by 40%.

Figure 5 shows testosterone production by Leydig cells cultured short term, for 2 hours, in the absence (basal) or presence of maximally stimulating LH (100 ng/ml), dbcAMP (1 mM), 22-hydroxycholesterol (20 μ M), or

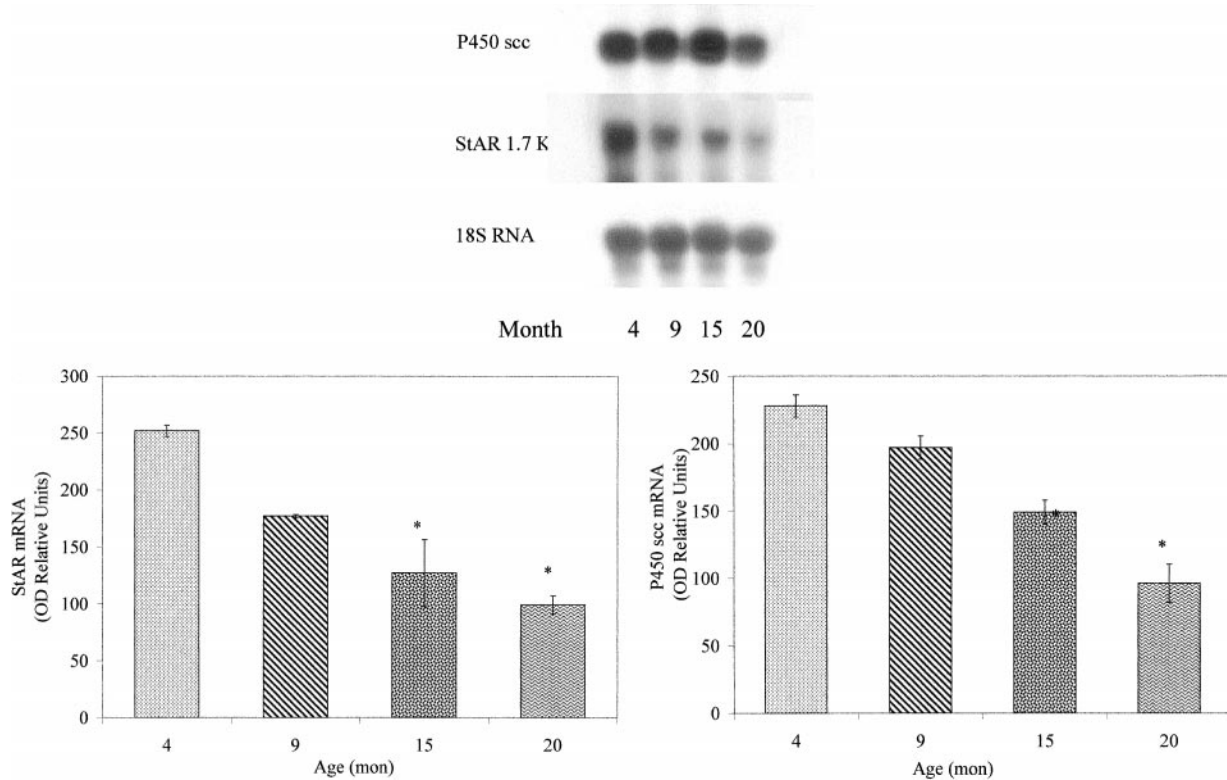


Figure 3. Northern blot analysis of total cellular RNA. **Top:** Representative Northern blots. For this study, 9–10 rats per age were used. For each age, Leydig cells were isolated from the testes groups of 3–4 rats and pooled before RNA isolations. This produced 3 RNA pools per age. **Bottom:** Bands corresponding to the 1.7-Kb transcript of steroidogenic acute regulatory protein mRNA and to P450 side-chain cleavage mRNA and 18S rRNA were quantified. The data are expressed as the mean \pm standard error of the mean of the 3 separate samples of RNA per age. *Significant difference from the 4-month-old values.

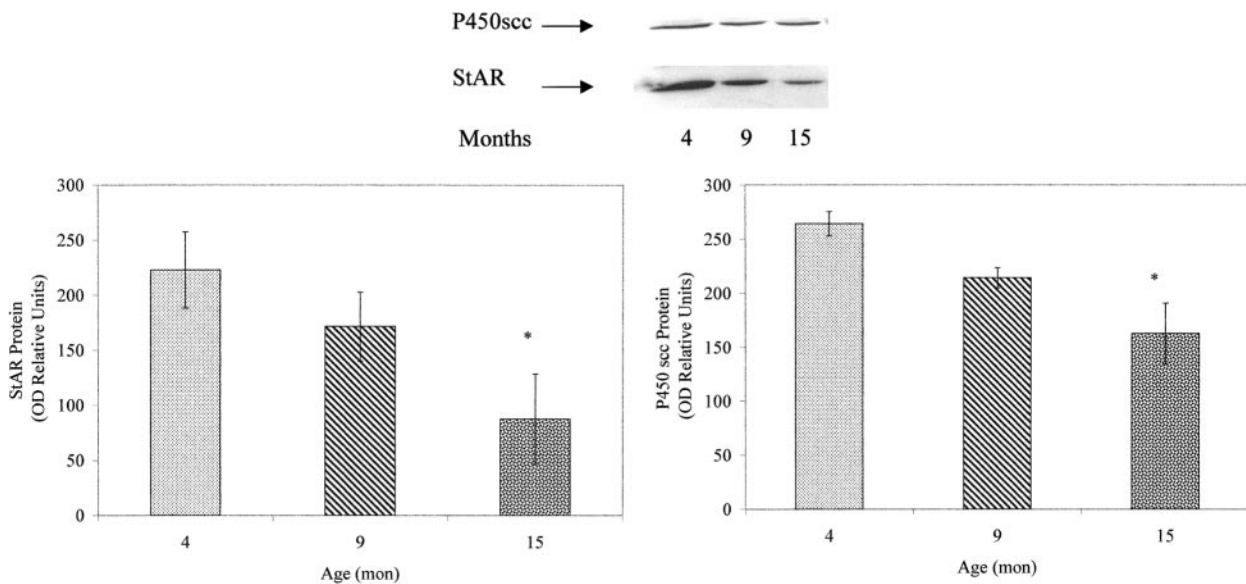


Figure 4. Western blot analysis. Leydig cells were isolated from 4-, 9-, and 15-month-old rats. **Top:** Immunospesific bands for steroidogenic acute regulatory protein and P450 side-chain cleavage protein are shown. Bands were quantified by computer-assisted image analysis. The data are expressed as integrated optical density (OD). Each data point represents the mean \pm standard error of the mean from 3 separate samples. *Significant difference from 4-month-old samples.

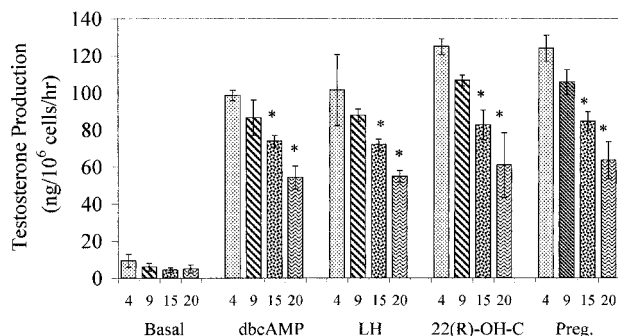


Figure 5. Testosterone production by isolated Leydig cells in vitro. Cells from 4–20-month-old rats were incubated with luteinizing hormone (100 ng/ml), dibutyl cyclic adenosine monophosphate (1 mM), 2R-hydroxycholesterol (20 μ M), or pregnenolone (2.5 μ M). The bars are the mean \pm standard error of the mean, generated as described for Figure 1. *Significant difference from the 4-month-old value.

pregnenolone (2.5 μ M). The rationale for this study was that dbcAMP bypasses LH receptor-G protein coupling; 22-hydroxycholesterol enters mitochondria directly, without the need for functioning transport proteins (StAR, PBR); and pregnenolone bypasses P450scc. With each of dbcAMP, 22-hydroxycholesterol, and pregnenolone, significant age-related declines in testosterone production were seen at 15 months, with further declines at 20 months. As with LH, reductions were seen at 9 months that were not significant.

Discussion

Previous studies have shown that the capacity of Leydig cells from aged Brown Norway rats to produce testosterone in response to LH stimulation is reduced in comparison to Leydig cells from young rats (Chen et al, 1994, 1996). The transport of cholesterol from intracellular stores to the inner mitochondrial membrane, a process in which StAR (Crivello and Jefcoate, 1980; Privalle et al, 1983; Stocco, 1996, 1999) and PBR (Culty et al, 2002; Liu et al, 2003) are integrally involved, and the subsequent conversion of cholesterol to pregnenolone on the inner membrane of the mitochondria, the site of P450scc, are considered to be the primary points of control in steroidogenesis (Farkash et al, 1986; Saez, 1994). Not surprisingly, therefore, aged cells have been shown to be characterized by reduced PBR (Culty et al, 2002), StAR, and P450scc (Luo et al, 2001). Reduced StAR suggests that there may be reduced cholesterol transfer to the P450scc enzyme within the mitochondria of old Leydig cells (Leers-Sucheta et al, 1999), and therefore compromise of StAR may play a role in the mechanism by which reductions occur in Leydig cell steroidogenesis with aging. Previous studies likewise have shown that P450scc also is reduced in old cells, and, indeed, that age-related

reductions occur in each of the steroidogenic enzymes downstream from P450scc, including 3 β -HSD, P450c17, and 17 β -HSD (Luo et al, 1996). Compromise of any 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.

A major objective of the present study was to determine the ages at which critical changes related to steroidogenesis are initiated in aging Leydig cells in relationship to reduced steroidogenesis. We focused on cAMP production, StAR, and P450scc. Significant reduction in the ability of the Leydig cells to produce testosterone, as assessed by their response to short-term (2 hour) exposure to maximally stimulating LH, was first seen in cells isolated from rats of 15 months of age. LH-stimulated intracellular cAMP levels were found to decline significantly by 9 months, before significant reductions in testosterone production were seen. In part, these results support the contention (Catt et al, 1980; Liu et al, 2003) that testosterone production can be maximal in the face of reduced cAMP. Further reductions in cAMP were seen through 15 and 20 months, along with decreases in testosterone. The observation that aging Leydig cells produce less cAMP than young cells suggests that the old cells have defects in the LH-cAMP signaling cascade.

Short-term (2 hours) exposure of Leydig cells to dbcAMP, which bypasses LH receptor-G protein coupling, is used to assess steps of the steroidogenic pathway beyond this point. Likewise, culturing the cells with 22-hydroxycholesterol, which enters mitochondria directly and thus obviates the need for functioning transport proteins (StAR, PBR), or pregnenolone, which bypasses P450scc, is used to examine the possibility of downstream deficits. Significant age-related declines in testosterone production were seen in each case. The results indicate that, as expected, aging cells have deficits at multiple steps along the steroidogenic pathway. It should be pointed out that exposure of the cells for 2 hours to LH, dbcAMP, 22-hydroxycholesterol, or pregnenolone is too short a time for steroidogenic function to be restored, and therefore this study was conducted to describe existing defects.

Given the critical role played by LH-stimulated cAMP in steroidogenesis (Dufau et al, 1980), it seemed reasonable to hypothesize that if cAMP production was reduced to sufficiently low levels, the result would be reduced testosterone production. In fact, significant decreases were seen in cAMP production at age 9 months, preceding decreases in StAR and P450scc mRNA, StAR and P450scc protein levels, and testosterone production. These observations suggested that changes in StAR and P450scc, and thus in testosterone production, might occur as a consequence of reduced cAMP. This hypothesis is supported by

a recently completed study in our lab in which aged Leydig cells were cultured with dbcAMP, a membrane-permeable cAMP agonist that bypasses the LH receptor-adenylyl cyclase cascade (Chen et al, 2004). In that study, long-term (3 day) culture restored the ability of Leydig cells from the testes of aged (21–24-month-old) rats to produce testosterone at the high level of cells from 4-month-old rats, and thus reversed the steroidogenic deficits of aging Leydig cells. Accompanying the restoration of testosterone production, StAR and P450_{scc} also were restored to the levels of young cells. These results are consistent with the hypothesis that the many changes in the steroidogenic pathway that accompany aging probably do not occur independently of one another, but rather that there is an initiating, early event (or events), perhaps reduced cAMP production, that ultimately leads to coordination of downstream changes in the remainder of the steroidogenic machinery, and thereby to reduced testosterone production.

The mechanism by which changes occur in aging Leydig cells remains uncertain. Reduced LH levels essentially have been ruled out (Chen et al, 1994, 1996; Gruenewald et al, 1994; Bonavera et al, 1997, 1998; Grzywacz et al, 1998). Although aged cells have fewer LH receptors than young cells, this is unlikely to explain the decreased cAMP production (Chen et al, 2002). If LH receptor coupling to adenylyl cyclase through G proteins was compromised, reduced cAMP would result (Dufau et al, 1980), and this certainly is a possibility. There is extensive evidence that free radical damage may contribute to cell aging (Pacifci and Davies, 1991; Knight, 2000). We have reported that aged Leydig cells produce more reactive oxygen than young cells (Chen et al, 2001), and unpublished studies from our lab indicate that the major scavengers of reactive oxygen (superoxide dismutase [SOD] I and II, glutathione peroxidase, and glutathione) all are reduced in aged cells. In combination, increased reactive oxygen production and a decreased ability of the cells to rid themselves of potentially damaging reactive oxygen species could lead to free radical damage. If free radical damage affected membrane fluidity (Vlasova, 2000; Karbownik et al, 2001), the LH receptor-G protein-adenylyl cyclase coupling cascades probably would become less efficient (Kolena et al, 1986), and this could disrupt LH-stimulated cAMP production (Wu et al, 1993). This is an appealing hypothesis to explain how aging might result in reduced intracellular cAMP, which, in turn, could result in reductions in cholesterol transport and/or metabolism and thus in reduced testosterone production.

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