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Cholesterol Transport, Peripheral Benzodiazepine Receptor, and Steroidogenesis in Aging Leydig Cells

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ABSTRACT: The cellular mechanisms responsible for age-related decline in the ability of Leydig cells to produce testosterone are not yet fully understood. The decline in testosterone production could result from a reduction in the Leydig cell enzymatic activities mediating testosterone synthesis, the amount of substrate available for these enzymes, or both. In the present study, we examined the effect of age on a critical early step in the steroidogenic pathway, the transport of cholesterol into mitochondria. Leydig cells were isolated from the testes of young and old Brown Norway rats and incubated with human chorionic gonadotropin (hCG) and the side-chain cleavage cytochrome $P450_{scc}$ inhibitor aminoglutethimide (AMG). Mitochondria were isolated from these cells in the presence of AMG. Upon removal of AMG, the mitochondria from old cells produced 80% less steroid than those from young cells, only a fraction of which could be accounted for by a decrease in $P450_{scc}$ activity. These results suggest that the accumulation of hormonally recruited cholesterol into mitochondria is defective in old Leydig cells. With this in mind, we turned our attention to peripheral benzodiazepine receptor (PBR), a mitochondrial cholesterol-binding protein known to be involved in mediating cholesterol transport. PBR messenger RNA (mRNA) and protein expression were decreased in old cells. Moreover, both the dissociation constant (Kd) and the number of binding sites (Bmax) of the PBR were decreased in the old cells by 50% and 30%, respectively. Taken together, these results suggest that alterations in cholesterol transport and in PBR may play critical roles in age-related decreases in testosterone production in Brown Norway rat Leydig cells.

Key words: Mitochondria, steroidogenic acute regulatory protein.
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The decline of circulating testosterone in aging men has been associated with various symptoms, including reduced stamina, erectile dysfunction, mood disturbance, memory loss, reduced muscle mass and strength, and altered fat distribution (Falahati-Nini et al, 2000; Jankowska et al, 2000; Kenny et al, 2000; Morley, 2000, 2001; van den Beld et al, 2000), as well as more troublesome pathologies such as osteoporosis and anemia (Bain, 2001). It has been shown that although there is a malfunction of the hypothalamic-pituitary axis in men, the decrease in testosterone is likely to involve direct alterations of testicular Leydig cell function (Gruenewald et al, 1994; Bonavera et al, 1997; Zirkin and Chen, 2000).

The Brown Norway rat presents an age-related primary testicular deficit similar to that observed in the human (Zirkin and Chen, 2000). In this rat, Leydig cell numbers do not change with aging. Rather, the capacity of old Leydig cells to produce testosterone is reduced (Chen et al, 1994, 1996; Luo et al, 1998; Zirkin and Chen, 2000). A decrease in Leydig cell steroidogenesis could result from a reduction in any of the enzymatic activities involved in testosterone synthesis (Hall, 1984, 1998) or could reflect a decrease in the amount of substrate available for these enzymes to form testosterone. Previous studies have shown that all enzymes involved in the synthesis of testosterone are decreased with aging (Luo et al, 1996, 2001). More recently, we reported that both the side-chain cleavage cytochrome $P450_{scc}$, responsible for the first step of steroidogenesis (Hall, 1984), and the steroidogenic acute regulatory protein (StAR), a hormone-induced protein considered to be involved in mediating cholesterol transport into the mitochondria (Clark et al, 1994), were decreased in Leydig cells from old Brown Norway rats compared to young rats (Luo et al, 2001). However, hormonal stimulation resulted in increases of testosterone production and StAR protein expression in cells from both old and young rats, indicating that the old Leydig cells have not lost the ability to respond to hormones (Luo et al, 2001). This further suggested that the net testosterone decrease observed might have additional causes, such as a deficit in the basal/unstimulated pool of cholesterol available for steroidogenesis.

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Since the limiting step of the steroidogenic cascade has been shown to be the transport of cholesterol to $P450_{scc}$, the first enzyme of the cascade (Crivello and Jefcoate, 1980; Privalle et al, 1983), it is easy to envision that the alteration of either the pool of cholesterol involved in this process or the intramitochondrial transport of cholesterol could perturb the steroidogenic process. In the present study, we compared the size of the mitochondrial cholesterol pool available for pregnenolone formation by young and old cells, the steroidogenenic response of the cells in the presence of nonlimiting levels of cholesterol, and the expression of peripheral benzodiazepine receptor (PBR), a high-affinity mitochondrial cholesterol-binding protein (Lacapère et al, 2001; Li et al, 2001) also known to mediate cholesterol transport in steroidogenic tissues (Krueger and Papadopoulos, 1990; Papadopoulos, 1993; Li and Papadopoulos, 1998). We report that aging is associated with decreases in the cholesterol stores available for steroidogenesis and in the expression of PBR protein, suggesting that these 2 events contribute to the testosterone decrease observed in aging.

Materials and Methods

Chemicals

R(+)-*p*-Aminoglutethimide (AMG) tartrate and PK 11195 were purchased from SIGMA/Research Biochemical International, Inc (Natick, Mass). [N-methyl-^{3}H]PK 11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinolinecarboxamide; sp act 75.00 Ci/mmol), [7^{3}H(N)]pregnenolone (sp act 21.1 Ci/mmol), and [1,2,6,7-^{3}H (N)]testosterone (sp act 96.50 Ci/mmol) were obtained from New England Nuclear Life Sciences (Wilmington, Del). 22R-Hydroxycholesterol and other steroids, sodium isocitrate, triethanolamine, dibutyryl cyclic adenosine monophosphate (dbcAMP), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Corporation (St Louis, Mo). Trilostane (4α,5-epoxy-17α-hydroxy-3-keto-5α-androstan-2α-carbonitrile) was a gift from Stegram Pharmaceuticals (Sussex, United Kingdom). SU-10603, an inhibitor of P450c17 enzyme, was a gift from CIBA-GEIGY (Suffern, NY). Purified human chorionic gonadotropin (hCG; batch CR-125 of biological potency 11 900 IU/mg) was a gift from Dr A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) National Hormone & Pituitary Program at the National Institutes of Health (Bethesda, Md). Antibodies to pregnenolone and testosterone were obtained from ICN Pharmaceuticals Inc (Costa Mesa, Calif). The antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Trevigen (Gaithersburg, Md). Cell culture supplies were purchased from Life Technologies Inc (Grand Island, NY). Percoll was purchased from Pharmacia (Piscataway, NJ). All other chemicals were of analytical quality and were obtained from various commercial sources.

Animals

Adult male Brown Norway rats aged 4 (young) or 24 (old) months were obtained through the National Institute on Aging, supplied by Harlan Sprague-Dawley Inc (Indianapolis, Ind), and housed in controlled light and temperature conditions as previously described (Luo et al, 2001).

Isolation of Leydig Cells

Leydig cells from young and old rats were isolated and purified by Percoll gradient and, in some studies, by centrifugal elutriation and Percoll gradient (Klinefelter et al, 1987). The purity of the cells, assessed by staining for 3β-hydroxysteroid dehydrogenase, was about 85% after Percoll gradient alone and was greater than 93% after a combination of Percoll gradient and centrifugal elutriation. This was true for both young and old cells. Cells from pooled testes from up to 5 rats were purified as 1 individual sample.

Mitochondrial Preparation

Mitochondria were prepared as previously described (Krueger and Papadopoulos, 1990). All steps of the procedure were done at 4°C. Briefly, aliquots of purified Leydig cells were washed in phosphate-buffered saline (PBS), resuspended in buffer A (50 mM Tris-HCl [pH 7.2], 250 mM sucrose), and homogenized with an electric Teflon-glass homogenizer. The homogenate was centrifuged at 800 × *g* for 10 minutes, the supernatant was collected, and the pellet was homogenized and centrifuged again. The 2 supernatants were pooled and centrifuged at 10 000 × *g* for 15 minutes. The resulting mitochondrial pellet was washed twice in buffer B (10 mM potassium phosphate [pH 7.4], 0.25 M sucrose, 5 mM MgCl2, 20 mM KCl, and 15 mM triethanolamine-HCl) containing 5 μM trilostane, which, by inhibiting 3β-hydroxysteroid dehydrogenase, prevents pregnenolone metabolism (Potts et al, 1978). The mitochondrial preparations were assayed for protein concentration and were used in steroidogenesis and PBR binding experiments. Each mitochondrial sample was prepared from Leydig cells obtained from the pooled testes of 2–5 rats in order to obtain purified mitochondrial aliquots containing at least 500 μg protein/mL.

Intact Cell Steroidogenesis

Leydig cells were suspended in ice-cold Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and they were then incubated for 2 hours with the agents to be tested in a 37°C shaking water bath, in the presence or absence of 10 μM trilostane plus 5 μM SU-10603 (Gower, 1974). The reactions were stopped by freezing the samples at −20°C for later determination of pregnenolone or testosterone concentration by radioimmunoassay.

Measurement of Cholesterol Accumulation Upon Hormonal Stimulation

In these experiments, intact Leydig cells were initially incubated for 2 hours at 37°C in the presence of 500 μM AMG, a specific inhibitor of $P450_{scc}$, and in the presence or absence of 50 ng/mL hCG, according to a protocol previously described (Boujrad et al, 1994). The AMG-mediated blocking of cholesterol metabolism upstream of the $P450_{scc}$ reaction results in the accumulation of cholesterol in the mitochondrial membranes, including the basal pools and those transferred to the mitochondria upon hor-

monal treatment. The cells were then washed twice with ice-cold PBS containing AMG and further processed for mitochondrial preparations as described above, except that AMG was included in every step until the last centrifugation. At this stage, AMG was removed and, after a final wash of the pellet in AMG-free buffer, the mitochondria were resuspended and incubated for 20 minutes at 37°C in the presence of 10 μM trilostane, 15 mM isocitric acid, and 0.5 mM NADP in order to allow the $P450_{scc}$ to function. Steroids were then extracted and measured by radioimmunoassay. The difference in pregnenolone formed between the aliquots from cells incubated with or without hCG corresponds to the cholesterol that was accumulated during stimulation and is proportional to the cholesterol transport.

Measurement of Mitochondrial Pregnenolone Synthesis

Mitochondria were resuspended in buffer B at a final concentration of 0.5–1.0 mg/mL of protein. In some experiments, 22R-hydroxycholesterol was added to the samples. The reaction was started by the addition of isocitric acid and NADP (at 15- and 0.5-mM final concentrations, respectively) in a final volume of 250 μL. After a 20-minute incubation at 37°C, the reaction was stopped by adding 100 μL of ethanol containing 2000 counts/min (cpm) of [^{3}H] pregnenolone as a recovery marker, followed by 1 mL of diethyl ether. After extraction, the organic phase was collected and evaporated to dryness. Pregnenolone was measured by a specific radioimmunoassay.

Radioimmunoassays

Pregnenolone and testosterone productions were measured using specific radioimmunoassays as previously described (Papadopoulos et al, 1990; Boujrad et al, 1994), following the conditions recommended by the supplier of the antibodies. The recoveries were determined and used to correct the results. Analysis of the radioimmunoassay data was performed using the MultiCalc Software from EG & G Wallac Inc (Gaithersburg, Md).

Radioligand-Binding Assays

The binding experiments were performed as previously described (Papadopoulos et al, 1990). Briefly, each individual sample of Leydig cells was purified from the pooled testes of 3 rats, either young or old, and 3 independent sets of such pools were used in each experiment. The purified cells were washed twice with ice-cold PBS and homogenized in PBS with a Teflon-glass homogenizer. Aliquots of cell lysate containing 20 μg protein/sample were incubated at 4°C in the presence of 0.03–20 nM [^{3}H]PK 11195 in a final incubation volume of 0.3 mL. Nonspecific binding was determined by adding 200-fold excess of unlabeled ligand in some samples. After a 90-minute incubation, assays were stopped by filtration through Whatman GF/B filters (Brandel, Gaithersburg, Md) equilibrated in 0.1% polyethyleneimine and washed with 20 mL ice-cold PBS. Radioactivity trapped on the filters was determined by liquid scintillation counting. The dissociation constant (Kd) and the number of binding sites (Bmax) were determined by Scatchard plot analysis of the saturation isotherms generated using the LIGAND program (KELL, version 4.0, Biosoft Inc, Ferguson, Mo) (Munson and Rodbard, 1980).

Immunoblot (Western) Analysis

Whole cells or mitochondria were solubilized in Laemmli buffer, and the proteins, loaded either at equal cell number per lane or equal protein amount per lane, were fractionated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto nitrocellulose, as previously described (Li et al, 2001). Immunoblot analysis of membranes was performed using a rabbit anti-PBR antiserum prepared by sequential immunization with a peptide of PBR protein (conserved sequence) coupled to keyhole limpet hemocyanin (1:1000) and a goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:5000) (Li et al, 2001). Positive bands were then detected by chemiluminescence with the Renaissance kit (DuPont/NEN). Equal protein loading was assessed by reprobing the blots with an anti-GAPDH antiserum (1:1000; Trevigen). Densitometric analysis of the immunoreactive protein bands was performed using OptiQuant Software from Packard Bioscience (Meriden, Conn).

RNA Blot (Northern) Analysis

Total RNA was isolated from freshly isolated young or old Leydig cells as previously described (Chomczynski and Sacchi, 1987; Luo et al, 2001). The RNA extraction was carried out in the presence of a known amount of ^{35}S-labeled RNA as an internal standard, and the results were normalized for the variations of RNA recovery. Equal numbers of young and old cells were loaded onto denaturing 1.2% agarose gels and electrophoresed. The gels were blotted by capillary transfer onto a nylon membrane and were further hybridized with a ^{32}P-labeled complementary DNA (cDNA) probe for PBR. The cDNA of the full-length mouse PBR was labeled with deoxycytidine 5′-[α-^{32}P] triphosphate (dCTP) at ca 10^8 cpm/μg DNA using a random primer synthesis method.

Protein Measurement

Protein levels were measured by the Bradford method (Bradford, 1976) using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, Calif) with BSA as a standard.

Statistical Analysis

Results are presented as mean plus or minus standard error of the mean of up to 4 independent experiments. To determine if the means of specific parameters differed significantly between young and old rats, they were compared with unpaired *t* tests using the InStat program (version 3.0) from GraphPad Software, Inc (San Diego, Calif). The Kd and Bmax values for PBR were compared between young and old rats with the Mann-Whitney *U* test, and multiple groups were compared by one-way analysis of variance, both using InStat. The changes observed with aging were considered statistically significant for P less than .05.

Results

Comparison of the Steroidogenic Activity of Leydig Cells From Young and Old Rats

Leydig cells from young and old rats were incubated for 2 hours with hCG, dbcAMP, or 22*R*-hydroxycholesterol,

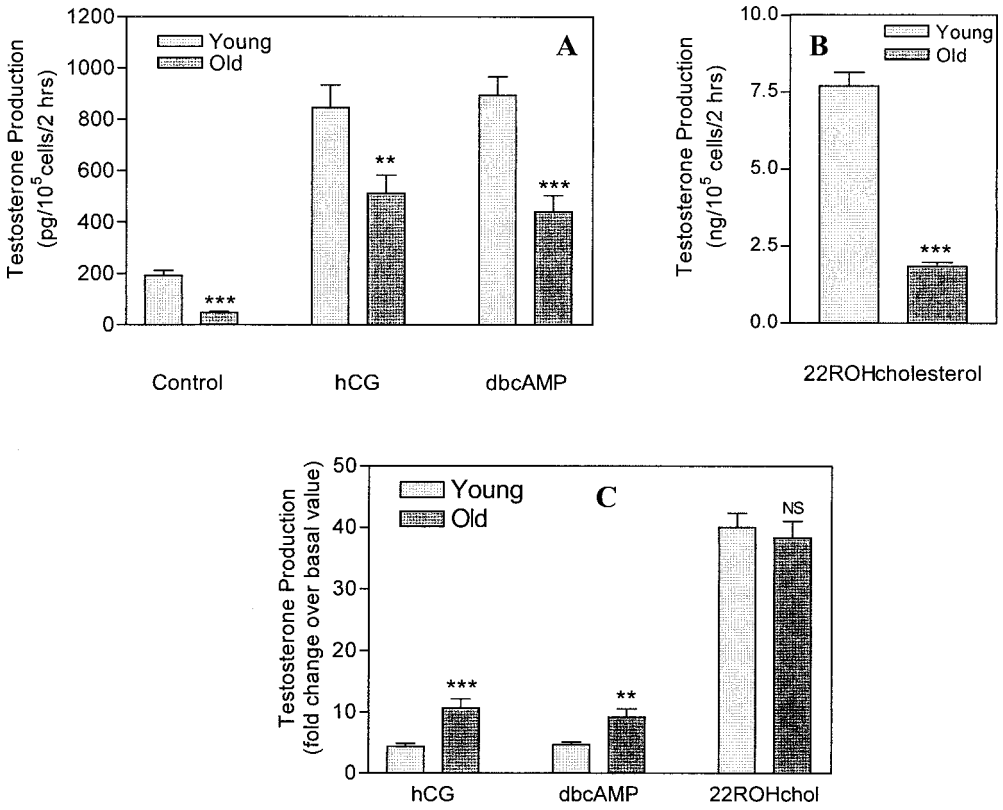

Figure 1. Effect of aging on Leydig cell steroidogenesis. Intact Leydig cells from young and old rats were purified and then incubated for 2 hours with human chorionic gonadotropin (hCG), dibutyryl cyclic adenosine monophosphate (dbcAMP), or 22*R*-hydroxycholesterol. Testosterone production was measured by radioimmunoassay as described in ''Materials and Methods.'' Testosterone production is expressed either as total steroid produced **(A, B)** or as fold changes against basal values **(C)**. Results represent mean plus or minus standard error of the mean of 4 independent experiments where each point was done in triplicate. Significantly different from young values: ***P* less than .01, ****P* less than .001. NS indicates nonsignificant.

a hydrosoluble substrate of the $P450_{scc}$ enzyme. As shown in Figure 1A, the basal production of testosterone (control) was 70% lower in Leydig cells from old than young rats; testosterone production in response to hCG was 30% lower in old than in young cells; and similar results were obtained in cells stimulated with dbcAMP. Figure 1B shows that testosterone production in the presence of 22*R*-hydroxycholesterol was 75% lower in old than in young cells but that, in both cases, it was 5–10 times greater than in hCG-stimulated cells. This suggests that the capacity of the cytochrome $P450_{scc}$ to metabolize cholesterol exceeds the size of the physiological cholesterol pool and provides evidence that the availability of substrate may be a limiting factor in the steroidogenic process. When the results were expressed as fold-increase over basal levels (Figure 1C), the difference between young and old Leydig cells disappeared, indicating that the steroidogenic enzymes were active in the old cells and thus suggesting that less enzyme or less substrate was present in old than in young cells. Experiments carried out on isolated mitochondria showed an effect of the exogenous substrate 22*R*-hydroxycholesterol on pregnenolone synthesis similar to that in whole cells (data not shown), confirming that $P450_{scc}$ remained functional in old cells.

In some experiments, cells were incubated in the presence of trilostane and SU-10603 to inhibit pregnenolone metabolism, in order to examine the $P450_{scc}$ activity by itself instead of the whole steroidogenic cascade. The difference in the levels of pregnenolone produced between old and young cells was similar to that found for testosterone produced in the absence of the inhibitors (data not shown).

Changes in Mitochondrial Cholesterol Accumulation With Aging

Since the availability of cholesterol appeared to be a critical factor in old cells, we performed further experiments to indirectly measure the size of the hormonally induced cholesterol pool. To this end, cells were incubated with hCG and the inhibitor of $P450_{scc}$, AMG, and mitochondria were then isolated from these cells in the presence of AMG. As shown in Figure 2, when young Leydig cells were incubated with AMG and hCG, there was a four- to sixfold increase in pregnenolone synthesis upon removal of the inhibitor from the mitochondria isolated from these

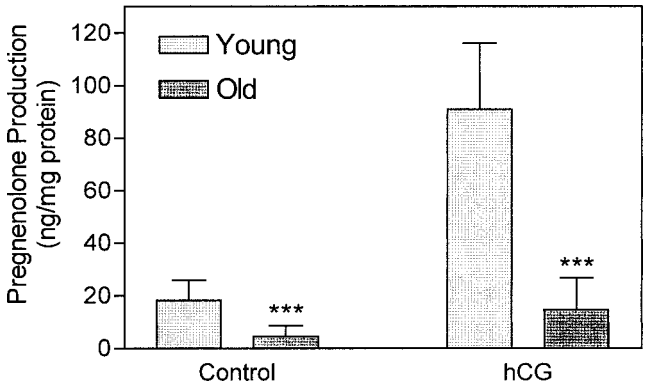

Figure 2. Effect of aging on mitochondrial cholesterol accumulation. Intact Leydig cells were initially incubated for 2 hours at 37°C in the presence of 500 μM aminoglutethimide (AMG) and in the presence or absence of 50 ng/mL human chorionic gonadotropin (hCG). Mitochondria were then isolated from these cells in the presence of AMG. AMG was finally removed, and the mitochondria were resuspended and incubated for 20 minutes at 37°C in the presence of 10 μM trilostane, 15 mM isocitric acid, and 0.5 mM NADP, in order to allow the side-chain cleavage cytochrome $P450_{scc}$ to function. Steroids were then extracted and measured by radioimmunoassay. The difference in pregnenolone formed in the samples from cells incubated with or without hCG is proportional to the cholesterol transport. Significantly different from young value: ***P less than .001.

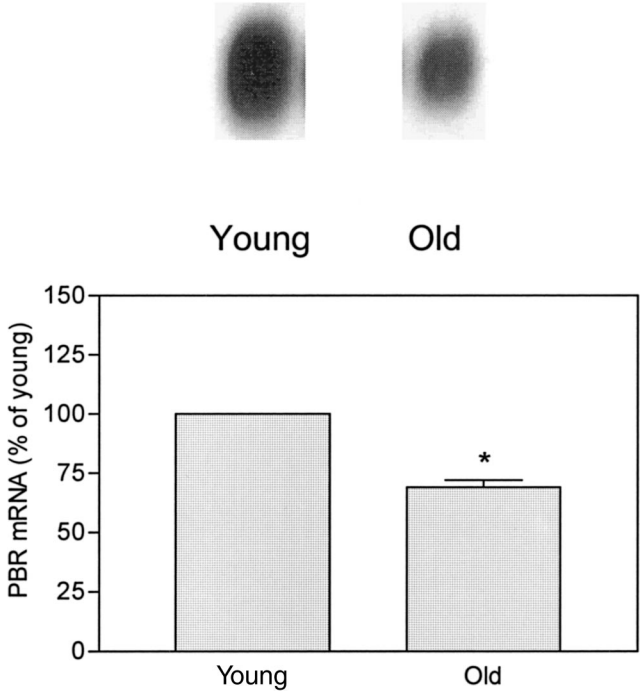

Figure 3. Effect of aging on peripheral benzodiazepine receptor (PBR) messenger RNA (mRNA) expression. Total RNA was isolated from equal numbers of young and old Leydig cells, and the RNA aliquots (equal cell numbers per lane) were loaded and electrophoresed on a denaturing 1.2% agarose gel. After blotting, the nylon membrane was hybridized with a ^{32}P-labeled complementary DNA (cDNA) probe for PBR (described in ''Materials and Methods''). The autoradiogram shows 1 representative experiment. The histogram shows the relative densitometric units expressed as percentages of the young samples and representing mean plus or minus standard error of the mean of 4 independent experiments. * Significantly different from young value: P less than .05.

cells, indicating that hCG stimulates an active accumulation of cholesterol into mitochondria. The same paradigm used on mitochondria from old rats showed that the hCG-induced pool of cholesterol available for $P450_{scc}$ after AMG removal was smaller than in young cells; there was only a two- to threefold increase in pregnenolone synthesis between mitochondria from control and hCG-treated old cells. This decrease in steroid formation in old vs young cells indicated that the hormonally activated cholesterol transport or loading to mitochondria was reduced by 80%–90% in old vs young cells when expressed as net steroid formed after subtracting the basal levels. It should also be noted that the basal levels of steroid formation were greatly diminished, by 60%–90%, in the mitochondria from old vs young cells, indicating an impairment of the homeostasis of cholesterol in the mitochondria of old rats.

Age-Dependent Changes in PBR Messenger RNA and Protein Expression

Considering the results presented above, showing that both basal and hormone-regulated levels of cholesterol were lower in old than in young cells, we compared the expression of the PBR, a mitochondrial protein known to mediate cholesterol transport in steroidogenic tissues, in young and old rat Leydig cells. As shown in Figure 3, PBR messenger RNA (mRNA) expression per cell was significantly lower (30% decrease) in Leydig cells from old than from young rats. For this study, total RNA from equal numbers of young and old cells was applied per lane. Aging also was accompanied by a significant decrease in the expression of the PBR protein (Figure 4). For this study, equal amounts of protein were applied per lane, and the Western blots were reprobed for GAPDH as a loading control. Thus, both PBR mRNA and protein expression in Leydig cells were affected by aging.

Age-Induced Changes in PBR Binding Sites

Radioligand-binding assays were performed on cell lysates from young and old Leydig cells in order to evaluate if aging has an effect on PBR expression and function. As shown in Figure 5A, the Kd of PBR was significantly decreased by 50% ($P < .05$) in old vs young cells. This increase of affinity of the receptor could be due to changes in protein structure or to changes in the environment of the receptor affecting its folding and conformation. Bmax values were also decreased (Figure 5B), but to a lesser extent, in old vs young cells. However, the changes in PBR protein could be greater than shown by the Bmax values. These values are expressed per milligram of total protein, and proteins other than PBR, such as the steroidogenic enzymes, are known to decrease with aging. Thus,

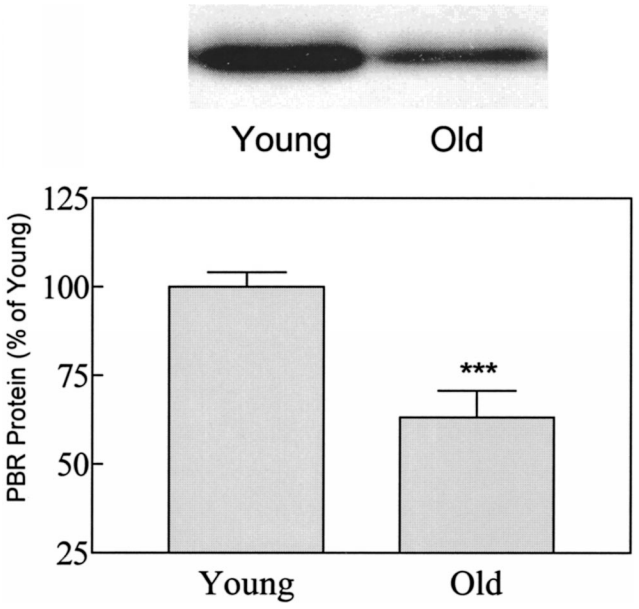

Figure 4. Effect of aging on peripheral benzodiazepine receptor (PBR) protein expression. Whole cells (or mitochondria) were solubilized in Laemmli buffer. Equal amounts of protein were loaded per lane and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblot detection of PBR using a rabbit anti-PBR antiserum. Blots were reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Positive bands were detected by chemiluminescence and quantified by densitometric analysis as described in ''Materials and Methods.'' Top: immunoreactive bands of PBR and GAPDH in samples from a representative experiment. Bottom: histogram showing the relative densitometric units expressed as percentages of the young samples and representing mean plus or minus standard error of the mean of 4 independent experiments. Significantly different from young value: ***P less than .001.

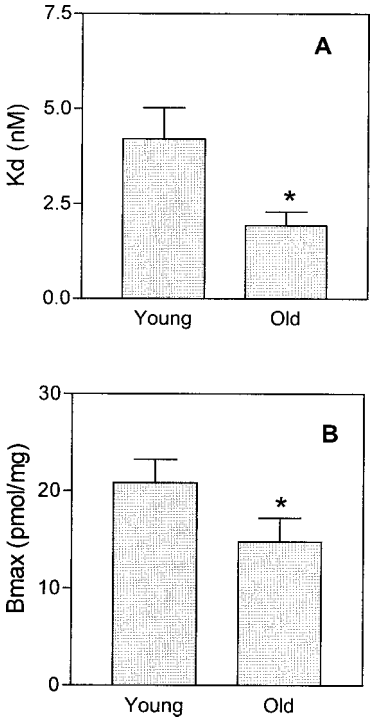

Figure 5. Effect of aging on peripheral benzodiazepine receptor (PBR) ligand-binding characteristics. The affinity (Kd) and the number of binding sites (Bmax) for each sample were determined through Scatchard analysis using the LIGAND program as described in ''Materials and Methods.'' Each value corresponds to the Scatchard analysis of Leydig cells purified from the pooled testes of 3 rats. * Significantly different from young value: P less than .05.

if a large number of proteins decrease significantly, the proportion represented by PBR could become larger, even though PBR itself might be decreased. Indeed, as shown by immunoblot analysis, the decrease in Bmax was less pronounced than the decrease found in the protein itself, indicating that age-related changes in the mitochondrial membrane may slightly improve the access of the ligand to the receptor, attenuating the decrease in the Bmax compared to the whole PBR protein.

Discussion

Because the medical community is actively debating the need to apply testosterone replacement treatment to elderly men (Janssens and Vanderschueren, 2000; Morley, 2000; Vermeulen, 2001), it has become critical to understand the cellular mechanisms underlying the decrease in testosterone that occurs with aging. As Leydig cells age, they produce less testosterone. Perturbation of any step in the sequence from luteinizing hormone binding to its receptor on Leydig cells through the steroidogenic reactions in the smooth endoplasmic reticulum could account for the observed age-related reductions in steroidogenesis. In fact, the activity, protein level, and mRNA level of $P450_{scc}$ and of each of the enzymatic reactions distal to this mitochondrial step (ie, in the smooth endoplasmic reticulum) are reduced in old Leydig cells (Luo et al, 1996, 2001).

The transport of cholesterol to the inner mitochondrial membrane is considered to be the rate-limiting step in steroidogenesis (Jefcoate et al, 1974; Hall, 1984). If the cholesterol available for translocation to the $P450_{scc}$ enzyme was reduced in old cells or if cholesterol movement to the $P450_{scc}$ enzyme was impeded, reduced testosterone production by the Leydig cells would be the likely outcome. We hypothesized that a reduction in cholesterol stores, or deficits in cholesterol transport, might indeed explain age-related decreases in testosterone production by the Leydig cells. To test this hypothesis, we first compared the steroidogenic capacity of young and old Leydig cells by measuring testosterone production by cells incubated with hCG or 22R-hydroxycholesterol, the latter a hydrosoluble substrate that easily enters mitochondria and thus bypasses the rate-limiting step of cholesterol transport between mitochondrial membranes (Hall, 1998). In both cases, the levels of testosterone produced by the old

cells were significantly reduced compared to young cells. Interestingly, however, testosterone production in the presence of 22*R*-hydroxycholesterol was dramatically increased in comparison to that stimulated by hCG. This was true of both young and old cells. Moreover, though the absolute amounts differed, the fold-increase in testosterone production from basal levels was at least equivalent in young and old cells. The difference observed in steroid production between young and old cells incubated with 22*R*-hydroxycholesterol suggests that the steroidogenic enzyme content per cell in the old cells is reduced and that this limits testosterone production. Indeed, previous studies have suggested that this is the case (Luo et al, 1996; Zirkin and Chen, 2000). The observation that the mitochondria of old cells produce less pregnenolone than those of young cells indicates that $P450_{scc}$, in particular, is reduced. However, given that the fold-increase in testosterone production was at least equivalent in young and old cells, it seems clear that the individual molecules of cytochrome $P450_{scc}$ function normally in old cells when nonlimiting amounts of substrate are present.

These observations led us to hypothesize that aging might be accompanied by a defect in cholesterol transport to mitochondria, resulting in less cholesterol available to the cytochrome $P450_{scc}$ in old cells. To test this hypothesis, Leydig cells were incubated with hCG and the $P450_{scc}$ inhibitor AMG, leading to the accumulation of hormonally recruited cholesterol into mitochondrial membranes (Boujrad et al, 1994). This paradigm allows for an indirect quantification of the cholesterol stores used in steroid synthesis, since the measurement of mitochondrial steroidogenesis after AMG removal is proportional to the cholesterol pool available for $P450_{scc}$. This experiment revealed a significant decrease in mitochondrial steroidogenesis in old vs young rats that was far greater than could be accounted for by a decrease in cytochrome $P450_{scc}$. These results indicated that, as hypothesized, there is a reduction in the amount of cholesterol transported into the mitochondria of old cells upon hormonal stimulation of the cells. Indeed, even without hormonal stimulation, significant differences were observed in steroid production between young and old cells. Taken together, these results suggest a potential defect in cholesterol transport in old Leydig cells.

The mechanism by which cholesterol translocation occurs continues to be debated, although it now seems clear that both StAR (Clark et al, 1994, 1995; Stocco and Clark, 1996) and PBR (Papadopoulos, 1993; Papadopoulos et al, 1997a,b) are involved. PBR, an integral outer mitochondrial membrane protein involved in the transport of cholesterol from the outer to the inner mitochondrial membranes in steroidogenic tissues (Krueger and Papadopoulos, 1990; Li and Papadopoulos, 1998), was recently shown to be a high-affinity cholesterol-binding protein (Lacapère et al, 2001; Li et al, 2001). Decreases in PBR expression have been shown in various models to correlate with decreases in steroid synthesis (Papadopoulos et al, 1997a,b). PBR is modified rapidly upon hormonal stimulation (Boujrad et al, 1994, 1996) and thus appears to play a role in the hormone-mediated response as well as in the maintenance of the basal pool of mitochondrial cholesterol (Papadopoulos et al, 1990).

In the present study, we found that both PBR mRNA and protein expression were reduced in old compared to young Leydig cells. Moreover, the receptor-binding experiments showed that both the Kd and Bmax of PBR were decreased in old cells. The increased affinity of the receptor could be due to changes in the protein structure, as well as to changes in the environment of the receptor affecting its folding and conformation. Although the ligand-biding site and the cholesterol-binding motif of PBR are localized on different domains of the receptor (Li and Papadopoulos, 1998; Li et al, 2001), a structural change in one area would most probably have consequences in other parts of the protein. Similarly, changes in the membrane environment adjacent to PBR would not only affect the ligand-binding site, but also other functional domains such as the cholesterol-binding site. Considering that PBR mediates the transfer of cholesterol from the outer to the inner mitochondrial membranes in steroidogenic cells (Krueger and Papadopoulos, 1990), any deficit in its expression/function should lead to a decrease in the cholesterol transport, and consequently, the cholesterol pool used during steroid synthesis. Indeed, the experiments performed in the presence of AMG confirmed the existence of a deficit in the basal and hormone-induced pools of cholesterol in mitochondria from old Leydig cells, which correlates with the decrease observed in PBR expression.

In conclusion, the results of the present study suggest that decreases in cholesterol transport to the mitochondria and changes in PBR may play significant roles in the decline of testosterone observed in aging. It now seems evident that a variety of factors are likely to be involved in age-related decreases in steroidogenesis, including a reduction in PBR and StAR and a reduction of the steroidogenic enzymes sequestered in the mitochondria and smooth endoplasmic reticulum. In this context, an association of PBR and StAR fusion proteins has recently been shown at the level of the mitochondrial membrane in a reconstituted cell model (West et al, 2001). Taken together, these observations suggest that correcting any one of these factors therapeutically may lead to an improvement in testosterone production but not a total recovery of the levels of testosterone present in young individuals.

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