Long-term suppression of Leydig cell steroidogenesis prevents Leydig cell aging

Haolin Chen and Barry R. Zirkin*

Division of Reproductive Biology, Department of Biochemistry and Molecular Biology, Johns Hopkins University, School of Hygiene and Public Health, 615 North Wolfe Street, Baltimore, MD 21205

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Male aging is accompanied by reduced testosterone production by the Leydig cells, the testosterone-producing cells of the testis. The mechanism by which this occurs is unknown. Based on the observations that reactive oxygen is capable of damaging components of the steroidogenic pathway and that reactive oxygen is produced during steroidogenesis itself, we hypothesized that long-term suppression of steroidogenesis might inhibit or prevent age-related deficits in Leydig cell testosterone production. To test this, we administered contraceptive doses of testosterone to groups of young (3 months old) and middle-aged (13 months old) Brown Norway rats via Silastic implants to suppress endogenous Leydig cell testosterone production. After 8 months, the implants were removed, which rapidly (days) restores the ability of the previously suppressed Leydig cells to produce testosterone. Two months after removing the implants, when the rats of the two groups were 13 and 23 months of age, respectively, the Leydig cells in both cases were found to produce testosterone at the high levels of young Leydig cells, whereas significantly lower levels were produced by the 23-month-old controls. Thus, by placing the Leydig cells in a state of steroidogenic ''hibernation,'' the reductions in Leydig cell testosterone production that invariably accompany aging did not occur. If hormonal contraception in the human functions the same way, the adverse consequences of reduced testosterone in later life (osteoporosis, reduced muscle mass, reduced libido, mood swings, etc.) might be delayed or prevented.

Human aging is accompanied by reduced testosterone concentration in the blood serum (1), a condition that has numerous potential adverse consequences including osteoporosis, reduced muscle strength, reduced libido, and mood changes (2). Although the mechanism by which testosterone concentration becomes reduced with age remains uncertain, recent studies of the Brown Norway rat, a strain in which aging of the male reproductive tract has a number of significant similarities to the human, have begun to shed light on this important issue (3–5). With aging, the capacity of the testes of these rats to produce testosterone declines significantly (3). This decline is associated with functional deficits of individual Leydig cells, the cells that are responsible for producing testosterone in the mammalian testis, and not with loss of Leydig cells (6). What might cause individual Leydig cells to become hypofunctional with respect to their ability to produce testosterone? Leydig cells depend on chronic stimulation by luteinizing hormone (LH) for the maintenance of their structure and steroidogenic function (7), suggesting that age-related reductions in steroidogenesis might result from deficits in LH. However, aging in the Brown Norway rat, as in the human, is not accompanied by reduced serum LH levels (6, 8), and, moreover, the administration of LH to aged rats failed to reverse the steroidogenic deficits of aged Leydig cells (9). Taken together, these observations suggest that LH understimulation probably is not responsible for age-associated changes in Leydig cell steroidogenesis.

The mechanisms that have been proposed to explain cellular aging in general can be classified into two major categories: programmed aging controlled by genes and unprogrammed aging resulting from cellular damage (10). The latter includes reactive oxygen-mediated damage to lipid, protein, and/or DNA (11). Reactive oxygen damage, if it occurred, would be expected to cause progressive deterioration of cellular function, potentially leading to functional senescence. Accumulated free radical damage seemed to us to be a plausible explanation for age-related functional deficits in the Leydig cells for two reasons: first, reactive oxygen has been shown to damage critical components of the steroidogenic pathway (12, 13), and, second, reactive oxygen species have been shown to be produced during steroidogenesis itself (13, 14). We reasoned that if, indeed, reactive oxygen species produced as a by-product of steroidogenesis were responsible for age-related reductions in testosterone production, the chronic suppression of steroidogenesis should diminish or prevent this consequence of aging.

We knew from previous studies that the administration of contraceptive doses of testosterone to rats, functioning through a negative feedback mechanism on pituitary LH, suppresses LH; that this, in turn, suppresses Leydig cell testosterone production; and that removal of the implants restores Leydig cell steroidogenesis (15, 16). Testosterone administration via Silastic implants, therefore, provided a means by which to reversibly suppress Leydig cell steroidogenesis, in effect placing the cells in a state of steroidogenic ''hibernation'' until removal of the implants. This process enabled us to examine the possibility that long-term suppression of steroidogenesis might prevent or delay the reduced steroidogenesis that accompanies Leydig cell aging.

Materials and Methods

Animals and Testosterone Implants. Adult male Brown Norway rats of 3 and 13 months of age were obtained through the National Institute on Aging, supplied by Charles River Breeding Laboratories. Rats were housed in virus-free facilities under controlled light (14-hr light/10-hr dark) and temperature (22°C) and had free access to rat chow and water. Implants (3-cm length) were made from Dow-Corning Medical Silastic tubing (1.98-mm i.d.), filled with testosterone (Steraloids, Wilton, NH), and sealed with Silastic medical adhesive A, as described (16). The release rate of testosterone from the capsules, determined by weighing the capsules before and after implantation, was 29 μg/cm per day.

Experimental Design. All procedures were in accord with protocols approved by the Johns Hopkins University Institutional Care and Use Committee. Young and middle-aged rats, of 3 and 13 months of age, respectively, received testosterone-filled implants (n = 16/group), and control rats of these ages (n = 8/group) received empty implants. Eight months later, when the rats were 11 and 21 months of ages, respectively, half of the rats that had received testosterone implants were killed by decapi-

Abbreviations: LH, luteinizing hormone; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17-KSR, 17-ketosteroid reductase; $P450_{scc}$, P450 cholesterol side-chain cleavage enzyme.

*To whom reprint requests should be addressed. E-mail: brzirkin@jhsph.edu.

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tation ($n = 8$/group), and the implants were removed from the remaining rats ($n = 8$/group). Two months later, when the latter rats had reached middle age (13 months) and old age (23 months), respectively, the rats were killed. Leydig cells were isolated from the testes by centrifugal elutriation and Percoll gradient centrifugation, as outlined below.

Leydig Cell Purification. Leydig cells were isolated as described (17). In brief, the testicular artery was cannulated and perfused with collagenase (1 mg/ml; Type 3; Worthington) in dissociation buffer (M-199 medium with 2.2 g/liter Hepes/1.0 g/liter BSA/25 mg/liter trypsin inhibitor/0.7 g/liter sodium bicarbonate, pH 7.4) to clear blood from the testes. Testes were decapsulated and digested in collagenase (0.25 mg/ml, 34°C) with shaking (90 cycles/min, 15 min). The dissociated cells then were subjected to centrifugal elutriation and Percoll gradient centrifugation purification, as described (17). The final purity of the Leydig cells, determined by staining the cells for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, consistently was about 95%.

Measurement of Leydig Cell Testosterone Production. Leydig cells were collected from testes of each of the treated and control rats and then pooled from groups of 2–3 rats. In this way, there were three pools of cells per treatment group. To measure the ability of the cells to produce testosterone, aliquots of 10^5 cells were incubated with maximally stimulating LH [100 ng/ml, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) oLH-26; National Hormone and Pituitary Program of NIDDK, Bethesda, MD] in 0.25 ml of medium 199 containing 1.0 g/liter BSA, 2.2 g/liter Hepes, and 1.2 g/liter sodium bicarbonate in 96-well plates for 2 hr at 34°C under 5% CO_2. Cells were run in triplicate. The testosterone concentration of the cells plus medium was assayed by RIA. Means were derived from the three pools of cells per treatment group and compared (see below).

Measurement of Steroidogenic Enzyme Activities. The activities of the steroidogenic enzymes sequestered in Leydig cell smooth endoplasmic reticulum (SER), namely, 3β-HSD, 17α-hydroxylase/C_{17-20} lyase ($P450_{17\alpha}$), and 17-ketosteroid reductase (17-KSR), and in mitochondria, namely P450 cholesterol side-chain cleavage enzyme ($P450_{scc}$), were measured by using the cultured Leydig cells (18, 19). To measure the activities of the SER enzymes, saturating concentrations of pregnenolone (P_5, 50 μM), progesterone (P_4, 12.5 μM), 17-hydroxyprogesterone ($17P_4$, 25 μM), or androstenedione (A, 12.5 μM) were added to the culture medium as substrate. The capacity of the Leydig cells to carry out specific steroidogenic reactions was calculated by summing all possible Δ^{4-3} ketosteroid products after a 2-hr incubation. For example, to measure the conversion of P_5 to P_4, P_5 was added to the medium and P_4 + 17 P_4 + A + T were measured and added together. To measure the conversion of P_4 to 17 P_4, P_4 was added to the medium and 17 P_4 + A + T were measured and added together. The conversions of the other intermediates were measured similarly. Δ^{4-3} Ketosteroid products were measured by using a HPLC/UV (240-nm wavelength) detection system. Mitochondrial $P450_{scc}$ activity was measured by quantifying isocaproic acid production after incubating the Leydig cells with 2 μM [26,27-^{3}H]25-hydroxy-cholesterol for 1 hr (19).

Western Blot Analysis of Steroidogenic Enzyme Proteins. Purified Leydig cells were solubilized (50 mM Tris, pH 6.8/5% 2-mercaptoethanol/2% SDS/10% glycerol/0.001% bromophenol blue), and proteins from equal numbers of cells were separated by 7.5% PAGE. The numbers of cells loaded for $P450_{scc}$, 3β-HSD, and $P450_{17\alpha}$ were 20×10^4, 5×10^4, and 20×10^4, respectively. The proteins then were electrotransferred onto nitrocellulose filters, and the filters were incubated with rabbit antisera to $P450_{scc}$ (20), 3β-HSD (21), or $P450_{17\alpha}$ (22). Subsequently, blots were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (Amersham Pharmacia), and an enhanced chemiluminescence (ECL) kit was used to detect the HRP-labeled protein according to the manufacturer's instructions (Amersham Pharmacia). Blots for each of the enzymes were repeated three times, using cells isolated and pooled from different groups of rats.

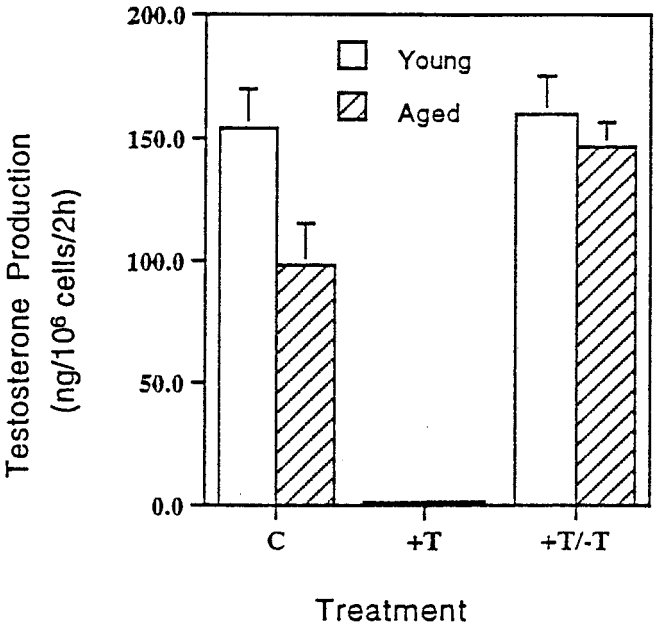

Fig. 1. The capacity of Leydig cells to produce testosterone, assayed *in vitro* under maximally stimulating LH. Within each experimental group of eight rats, there were three subgroups of 2–3 rats from which Leydig cells were isolated and pooled. The cells were run in triplicate. The graph shows the mean of the three subgroups ± SEM. C, Control rats of 3 and 13 months of age received empty implants for 8 months; the implants were removed; and 2 months later, when the rats were 13 (young) and 23 (aged) months of age, respectively, Leydig cells were isolated and assessed for their ability to produce testosterone. Leydig cells from the 23-month-old control rats produced significantly less testosterone than cells from the 13-month-old controls. +T, Leydig cells isolated from 11- and 21-month-old rats that had received exogenous testosterone for 8 months produced little testosterone. +T/−T, Two months after the implants were removed, when the rats were 13 and 23 months of age, respectively, the ability of the Leydig cells to produce testosterone was equivalent, in both cases at the high level of young controls.

Statistical Analyses. Data are expressed as the mean ± SEM. Statistical differences involving multiple group comparisons were determined by one-way ANOVA. If group differences were revealed by ANOVA ($P < 0.05$), differences between individual groups were determined with the Scheffe F test ($P < 0.05$).

Results

Testosterone Production. As shown in Fig. 1, Leydig cells from the testes of aged (23 months old) control rats produced significantly less testosterone than cells from the middle-aged (13 months old) controls. As expected from previous studies (15), the administration of testosterone-filled implants to rats of 3 and 13 months of age for 8 months suppressed Leydig cell testosterone production. Thus, Leydig cells from both the 11- and 21-month-old implanted rats produced very little testosterone. Two months after the implants were removed from the 11-month-old rats that had received implants when they were 3 months old, the ability of the Leydig cells to produce testosterone was at the high level of age-matched controls, a level comparable to that of young

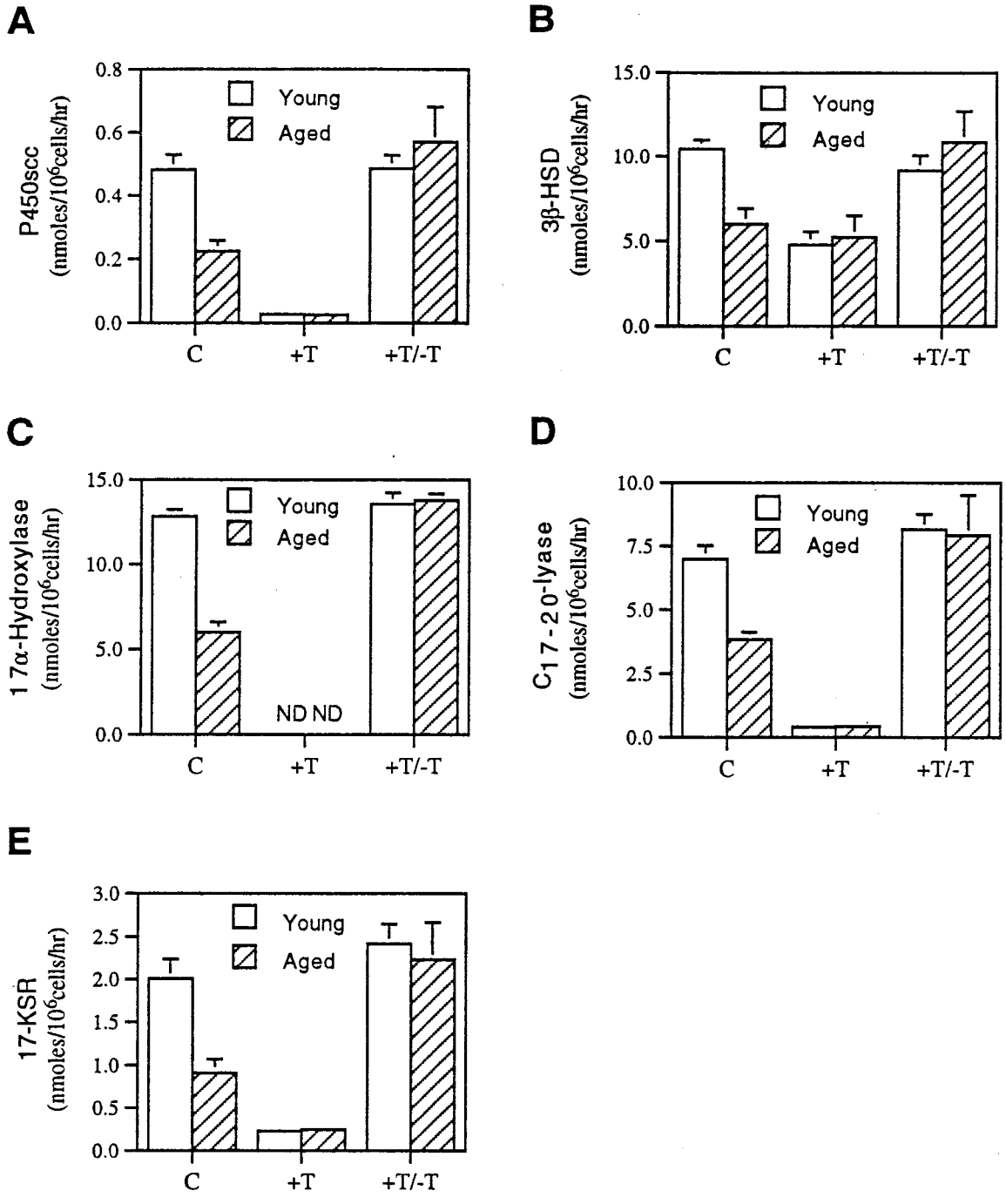

Fig. 2. Leydig cell steroidogenic enzyme activities, determined *in vitro*. C, Control rats of 3 and 13 months of age received empty implants for 8 months, the implants were removed, and 2 months later, Leydig cells were isolated and assessed for steroidogenic enzyme activities. The activities of each of $P450_{scc}$ (*A*), 3β-HSD (*B*), 17α-hydroxylase (*C*), C_{17-20} lyase (*D*), and 17-KSR (*E*) were significantly lower in Leydig cells from the 23-month-old compared with the 13-month-old control rats. +T, All the steroidogenic enzyme activities were reduced in Leydig cells isolated from 11- and 21-month-old rats that had received exogenous testosterone for 8 months. +T/−T, Two months after the implants were removed, when the rats were 13 and 23 months of age, respectively, the activities of each of the suppressed enzymes were at the high levels of 13-month-old control rats, in each case significantly higher than those of the 23-month-old controls. Means ± SEM are shown. ND, not detectable.

rats. Surprisingly, 2 months after the implants were removed from the 21-month-old rats, the ability of the Leydig cells to produce testosterone was at a level as high as that of young or middle-aged rats. Thus, suppression of steroidogenesis from age 13 to 21 months prevented the reduced steroidogenesis that accompanies aging.

Steroidogenic Enzymes: Analyses of Activities. The activities of the steroidogenic enzymes, from $P450_{scc}$ to 17-KSR, are shown in Fig. 2. The activities of each of $P450_{scc}$, 3β-HSD, 17α-hydroxylase, C_{17-20} lyase, and 17-KSR were significantly lower in Leydig cells from 23-month-old compared with the 13-month-old control rats. After 8 months of testosterone administration,

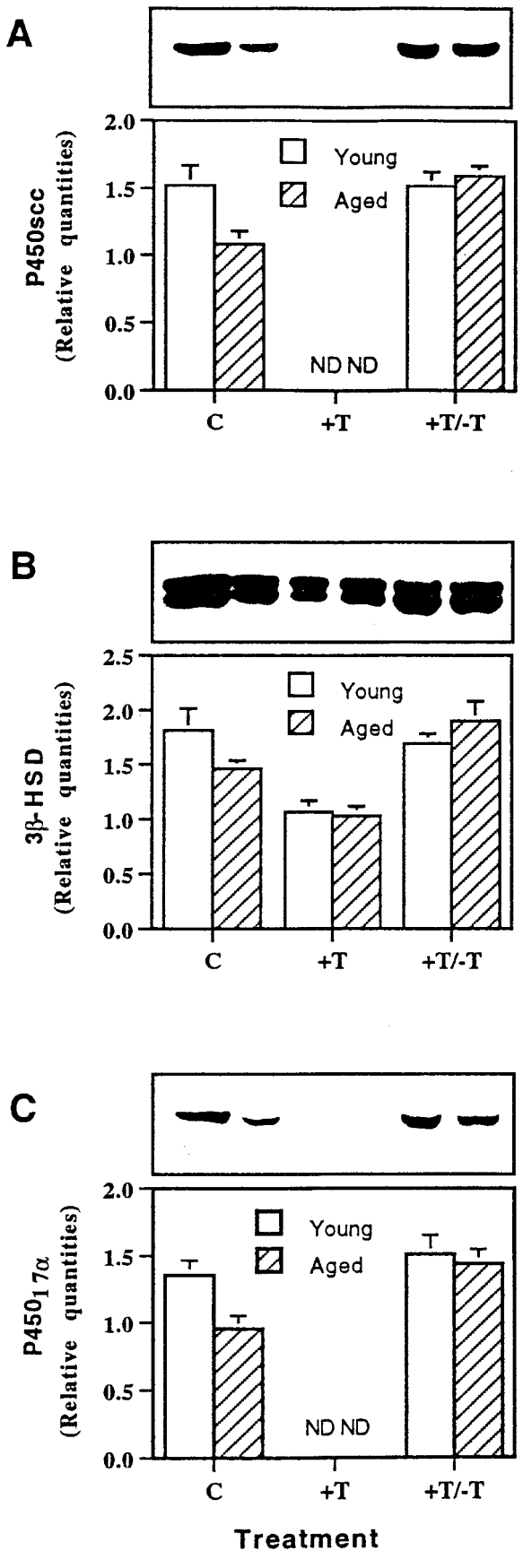

Fig. 3. Analysis of Leydig cell steroidogenic enzyme proteins by Western blot. Above each graph is a representative Western blot. Proteins ($P450_{scc}$, 3β-HSD, and $P450_{17a}$) were quantified by densitometric scanning of blots from three different pools of cells. C, Control rats of 3 and 13 months of age received empty implants for 8 months, the implants were removed, and 2 months later, when the rats were 13 and 23 months of age, respectively, Leydig cells were isolated and protein from equal numbers of cells was analyzed. The quantities of the $P450_{scc}$ (*A*), 3β-HSD (*B*), and $P450_{17a}$ (*C*) were reduced in cells of the 23-month-old compared with the 13-month-old rats. +T, After 8 months of testosterone treatment, all three enzymes were reduced, in the case of $P450_{scc}$ and $P450_{17a}$, to undetectable levels. +T/−T, Two months after the removal of the implants, when the rats were 13 and 23 months of age, respectively, the steroidogenic enzyme levels in both age groups were restored to those comparable to the 13-month-old controls, significantly higher than the 23-month-old controls. Means ± SEM are shown. ND, not detectable.

all the steroidogenic enzyme activities were reduced compared with the controls in both groups of rats. As also reported previously (15, 23), the response of 3β-HSD activity to exogenously administered testosterone was less than that of the other steroidogenic enzyme activities. Two months after the implants were removed from the 11- or 21-month-old rats, the activities of each of the previously suppressed enzymes were at the high levels of 13-month-old control rats, significantly higher than those of the 23-month-old controls. This finding is consistent with the ability of the cells to produce testosterone.

Steroidogenic Enzymes: Western Blot Analyses. Fig. 3 shows Western blots of the steroidogenic enzymes $P450_{scc}$, 3β-HSD, and $P450_{17\alpha}$, and quantitative analyses of these blots. In each case, the protein levels were consistent with the corresponding enzyme activities (Fig. 2). Thus, protein levels were lower in the 23-month-old compared with the 13-month-old controls. After 8 months of testosterone treatment, all three enzymes were reduced, in the case of $P450_{scc}$ and $P450_{17\alpha}$ protein to undetectable levels. Two months after the removal of the implants from the 11- or 21-month-old rats, the enzymes at both age groups were restored to levels comparable to those of the 13-month-old controls, significantly higher than 23-month-old controls. Again, this finding is consistent with the observation of testosterone production at young (high) levels.

Discussion

Long-Term, Reversible Suppression of Steroidogenesis Prevents Reduced Steroidogenesis. At the present time, the mechanism by which Leydig cells increasingly lose the ability to produce testosterone as they age is unknown. In other, far better-studied systems, however, age-related changes have been ascribed in part to reactive oxygen-mediated damage to lipid, protein, and/or DNA (11). Based on the observations that reactive oxygen is capable of damaging components of the steroidogenic pathway *in vitro* and that reactive oxygen is produced during steroidogenesis, it seemed plausible to us that the by-products of steroidogenesis itself might play a role in age-related reductions in Leydig cell steroidogenesis. We hypothesized that, if this were the case, long-term suppression of steroidogenesis should prevent or delay age-related reductions in steroidogenesis. Indeed, in the present study, we found that chronic suppression of Leydig cell steroidogenesis prevented the decreases in the capacity of the cells to produce testosterone that otherwise accompany aging. The results of measuring testosterone production were supported by measuring steroidogenic enzyme activities and protein content, which, in the present and previous (18) studies, have been shown to decrease as the ability of the Leydig cells to produce testosterone decreases during aging. Thus, the steroidogenic enzyme activities and proteins were restored to the high levels of young Leydig cells after chronic suppression of Leydig cell steroidogenesis whether the starting point was rats of 3 or 13 months of age.

By What Mechanism Does Suppression of Steroidogenesis Prevent/Delay Age-Related Reductions in Testosterone Production? Taken together, these results suggest that the chronic suppression of Leydig cell steroidogenesis, which, in effect, places the cells in steroidogenic "hibernation," in some way delays or prevents the reduced steroidogenesis that normally accompanies aging. We do not know for how long Leydig cells situated in an aged testis, and in the environment of an aged hypothalamic-pituitary axis, will continue to produce high (young) levels of testosterone. If the level of testosterone production remained high in the testes of aged rats, the conclusion would be that the aging of Leydig cells is intrinsic to the cells, not the result of extrinsic endocrine and/or paracrine effects. If, instead, Leydig cells in the aged testes show reduced steroidogenesis sooner after the removal of implants than those in the younger animals, one would conclude that there is an extrinsic component to Leydig cell aging.

The mechanism by which suppression of steroidogenesis results in delay or prevention of age-related reductions in the ability of Leydig cells to produce testosterone remains uncertain, though, as indicated above, an attractive possibility is that long-term suppression of steroidogenesis prevents free radical damage to the cells by suppressing reactive oxygen production during steroidogenesis itself. Indeed, as indicated above, there is evidence that reactive oxygen species are produced during Leydig cells steroidogenesis *in vitro* (12, 13) and *in vivo* (14) and that these species can damage critical cellular function, including the steroidogenic enzymes. However, there is no direct evidence showing that the reactive oxygen species, whether derived from steroidogenesis or elsewhere, are involved in Leydig cell aging, nor which cellular constituents (DNA? lipid? protein?) or processes are damaged by free radicals, if any.

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1. Chen, H., Luo, L. & Zirkin, B. R. (1996) in *The Leydig Cell*, eds. Payne, A. H., Hardy, M. P. & Russell, L. D. (Cache River, Vienna, IL), pp. 221–230.
2. Sternbach, H. (1998) *Am. J. Psychiatry* **155,** 1310–1318.
3. Zirkin, B. R., Santulli, R., Strandberg, J. D., Wright, W. W. & Ewing, L. L. (1993) *J. Androl.* **14,** 118–123.
4. Wang, C., Leung, A. & Sinha-Hikim, A. P. (1993) *Endocrinology* **133,** 2773–2781.
5. Gruenewald, D. A., Naai, M. A., Hess, D. L. & Matsumoto, A. M. (1994) *J. Gerontol.* **49,** B42–B50.
6. Chen, H., Hardy, M. P., Huhtaniemi, I. & Zirkin, B. R. (1994) *J. Androl.* **15,** 551–557.
7. Saez, J. M. (1994) *Endocr. Rev.* **15,** 574–626.
8. Chen, H., Huhtaniemi, I. & Zirkin, B. R. (1996) *Endocrinology* **137,** 3447–3452.
9. Grzywacz, F. W., Chen, H., Allegretti, J. & Zirkin, B. R. (1998) *J. Androl.* **19,** 625–630.
10. Davies, I. (1992) in *Textbook of Geriatric Medicine and Gerontology*, eds. Broolehurst, J. C., Tallis, R. C. & Fillit, H. M. (Churchill Livingstone, Edinburgh), pp. 26–60.
11. Stadtman, E. R. (1992) *Science* **257,** 1220–1224.
12. Quinn, P. G. & Payne, A. H. (1984) *J. Biol. Chem.* **259,** 4130–4135.
13. Quinn, P. G. & Payne, A. H. (1985) *J. Biol. Chem.* **260,** 2092–2099.
14. Peltola, V., Huhtaniemi, I., Metsa-Ketela, M. & Ahotupa, M. (1996) *Endocrinology* **137,** 105–112.
15. Keeney, D. S., Mendis-Handagama, S. M. L. C., Zirkin, B. R. & Ewing, L. L. (1988) *Endocrinology* **123,** 2906–2915.
16. Keeney, D. S., Sprando, R. L., Robaire, B., Zirkin, B. R. & Ewing, L. L. (1990) *J. Endocrinol.* **127,** 47–58.
17. Klinefelter, P. F., Hall, P. F. & Ewing, L. L. (1987) *Biol. Reprod.* **36,** 769–783.
18. Luo, L., Chen, H. & Zirkin, B. R. (1996) *J. Androl.* **17,** 509–515.
19. Georgiou, M., Perkins, L. M. & Payne, A. H. (1987) *Endocrinology* **121,** 1390–1399.
20. Gomberg-Malool, S., Ziv, R., Re'em, Y., Posner, I., Levitzki, A. & Orly, J. (1993) *Endocrinology* **132,** 362–370.
21. Doody, K. M., Carr, B. R., Rainey, W. E., Byrd, W., Murry, B. A., Strickler, R. C., Thomas, J. L. & Mason, J. I. (1990) *Endocrinology* **126,** 2487–2492.
22. Hales, D. B., Sha, L. & Payne, A. H. (1987) *J. Biol. Chem.* **262,** 11200–11206.
23. Wing, T.-Y., Ewing, L. L., Zegeye, B. & Zirkin, B. R. (1985) *Endocrinology* **117,** 1779–1787.