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Age-dependent changes in the function and morphology of mitochondria of rat adrenal zona fasciculata

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Summary. The function and morphology of adrenal zona-fasciculata (ZF) mitochondria were studied in 4-, 10- and 16-month-old rats, since in this species ageing causes a marked decline in glucocorticoid secretion coupled with high levels of circulating ACTH. Dispersed intact ZF cells displayed a significant age-dependent impairment of their basal pregnenolone (PREG) secretion, but isolated ZF mitochondria showed an increased capacity to convert cholesterol to PREG (the first rate-limiting step of steroid synthesis). These data are in keeping with the contention that the age-related deficit of rat ZF secretion is located prior to the activity of intramitochondrial cholesterol side-chain cleaving enzymes (cytochrome-$P450_{scc}$). Stereology showed a notable age-dependent increase in the number of mitochondria per unit cell-volume, coupled with a marked decrease in their average volume. The width of the mitochondrial intermembrane space remained unchanged, but its average volume strikingly decreased. This last finding fits well with the enhanced capacity of mitochondria to produce PREG, since intermembrane space is an acqueous barrier to the translocation of free cholesterol from the outer membrane to the cristae, where cytochrome-$P450_{scc}$ is located. In conclusion, the hypothesis is advanced that all these age-related functional and morphological mitochondrial changes are an ACTH-dependent compensatory response enabling ZF cells to partially counteract their decreased glucocorticoid secretory capacity, which in turn is due to the impaired utilization of intracytoplasmic stores of cholesterol esters.

Key words: Adrenal cortex, Ageing, Mitochondria, Rat, Sterology

Introduction

Many lines of evidence indicate that, in rats, the process of ageing is accompanied by a marked impairment in the adrenal glucocorticoid (corticosterone) secretory activity, with the consequent increase in the blood concentration of ACTH. The prolonged exposure to high levels of circulating ACTH provokes a notable hypertrophy of the zona fasciculata (ZF) and its cells, which, at least in part, is able to compensate the age-dependent secretory deficit (Malamed and Carsia, 1983; Popplewell et al., 1986; Reaven et al., 1988; Rebuffat et al., 1992). The decline in corticosterone secretion appears to be due to a deficit of steroidogenic machinery occurring prior to the conversion of cholesterol to pregnenolone (PREG), the rate-limiting step of steroidogenesis (for review, see Hanukoglu, 1992). In fact, the activity of intramitochondrial cholesterol side-chain cleaving enzymes (cytochrome-$P450_{scc}$) increased during ageing, and that of other enzymes (Δ^5-3ß-hydroxysteroid dehydrogenase-Δ^5/Δ^4-isomerase, and 11ß-hydroxylase) remains unchanged (Popplewell et al., 1987). Compelling evidence indicates that neutral cholesteryl-esterase activity steadily decreases until the 18th month of age (Popplewell and Azhar, 1987), and this is in keeping with the striking accumulation of cholesterol ester-storing lipid droplets occurring in adrenocortical cells of aged rats (Rebuffat et al., 1992).

The stimulation of cytochrome-$P450_{scc}$ activity is the main mechanism underlying the steroidogenic action of ACTH (for review, see Miller, 1988), and some investigations showed that the exposure to ACTH induces conformational changes in adrenocortical mitochondria, which may facilitate the transfer of free cholesterol from their outer to inner membranes, where cytochrome-$P450_{scc}$ is located (Lambeth and Stevens, 1984-85; Stevens et al., 1985; Boshier et al., 1990). Previously, it has been shown that the age-dependent (probably ACTH-induced) hypertrophy of rat ZF cells is coupled with a stereologically-demonstrable parallel increase in the volume per cell of the mitochondrial compartment, but the examination of the electron micrographs did not reveal subjectively appreciable alterations of mitochondria (Rebuffat et al., 1992).

It therefore seemed worthwhile to perform an accurate stereological and functional study of

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mitochondria of rat ZF cells during ageing.

Materials and methods

Male Wistar rats (Morini, Reggio Emilia, Italy) were used and housed 4 per cage at 20±2 °C on a 12:12h light-dark cycle. The rats were sacrificed at 4, 10 and 16 months of age. During the course of the study, all animals were in good health, without signs of mycoplasmosis. The rats were decapitated at 10:00 a.m.; the adrenal glands were promptly removed, freed of pericapsular fat and immediately employed for biochemical and morphological studies.

Biochemical studies

The adrenal glands of 16 rats from each age group were employed. The capsule was stripped to eliminate zona glomerulosa, the gland was bisected, and each adrenal half was enucleated to eliminate zonae reticularis and medullaris.

Preparation of dispersed cells

Dispersed ZF cells were obtained from the adrenal pairs of 8 rats from each age group by collagenase digestion and mechanical disaggregation (Szalay, 1981). Isolated cells were suspended in Medium 199 (DIFCO, Detroit, Mich., USA) and potassium-free Krebs-Ringer bicarbonate buffer, containing 5 mg/ml human serum albumin (Andreis et al., 1989). Aliquots of cell suspensions ($3x10^5$ cells/ml), obtained from the adrenal pair of each rat, were incubated in triplicate in the presence of 10 µM cyanoketone (WIN 24540; Sterling-Winthrop, Guilford, UK) to prevent further metabolism of PREG (Aguilera et al., 1981). The incubation was carried out in a shaking bath at 37 °C for 90 min, in an atmosphere of 95% O_2 and 5% CO_2.

Preparation of mitochondria

Mitochondria were isolated from the adrenal pairs of another 8 rats from each age group, according to Popplewell et al. (1987). Aliquots of mitochondrial preparations (200 µg/ml), obtained from the adrenal pair of each rat, were incubated in duplicate in the presence of 25-hydroxycholesterol (20 µg/ml) and 10 µM cyanoketone. The incubation was carried out at 37 °C for 15 min, and stopped by quick freezing at -70 °C.

PREG assay

PREG was extracted from the incubation media with dichloromethane; the extracts were washed twice with 0.1N NaOH and distilled water (2:1 v/v), and then evaporated to dryness under vacuum and redissolved in 50 µl methanol. PREG concentration was measured by HPLC (Perkin-Elmer, Norwalk, CT, USA), as previously described (Neri et al., 1993).

Morphological studies

The adrenal glands of 8 rats from each age group were processed for electron microscopy (Rebuffat et al., 1992). Thin (70 nm) sections were cut with an LKB Supernova ultramicrotome at the level of the middle portion of ZF. Thin sections were counterstained with lead-hydroxide and examined in a Hitachi H-300 electron microscope at a direct magnification of 7,000 or 20,000. The sampling procedure used to record electron micrographs for stereology was that described elsewhere (Belloni et al., 1990).

Determination of volume and surface densities (Vv and Sv) of mitochondria

Stereological analysis followed standard «differential point counting» and «linear intersection counting» practices for the determination of Vv and Sv, respectively (Weibel, 1979). The Vv of mitochondrial compartment in ZF cells (Vv_{mit}, $\mu m^3/\mu m^3$ of cell) was measured on electron micrographs at a final magnification of 21,000 (12 electron micrographs per rat). On electron micrographs at a final magnification of 70,000 (18 electron micrographs per rat), Vv and Sv of the following submitochondrial compartments (μm^3 or $\mu m^2/\mu m^3$ of mitochondria) were estimated; outer compartment (Vv_{oc}); matrix (Vv_{ma}); cristal space (Vv_{cs}); outer membrane (Sv_{om}); and inner (cristal) membrane (Sv_{im}) (Fig. 1).

Determination of average diameter, volume and numerical density (Nv) of mitochondria

The estimation of the average diameter of mitochondria ($\bar{D}_{mit}$) was based on the assumption that the shape of these organelles in rat ZF cells is spherical, since thin sections show circular or slightly elliptical profiles, with an average axial ratio near to unit (1.080-1.120). When elliptical profiles were encountered the arithmetic mean of the major and minor axes was taken as the circle diameter. The diameter distribution of mitochondrial profiles was determined on the electron micrographs at x 21,000, and from it the actual $\bar{D}_{mit}$ was calculated applying Schwartz's correction for spheres. Details of this statistical procedure are given in Nussdorfer et al. (1974). From $\bar{D}_{mit}$ the average volume of mitochondria ($\bar{V}_{mit}$) was calculated by the formula $4/3\pi(\bar{D}_{mit}/2)^3$. By knowing Vv_{mit} and $\bar{V}_{mit}$, Nv_{mit} (i.e. number of mitochondria per μm^3 of cell) was estimated. From $\bar{D}_{mit}$ the average surface area of outer mitochondrial membrane ($\bar{S}_{mit}$) was obtained by the formula $4\pi(\bar{D}_{mit}/2)^2$. From $\bar{V}_{mit}$ and $\bar{S}_{mit}$, Sv_{om} was recalculated; the estimates of Sv_{om} performed by the two techniques were in good agreement (differences less than 10%).

Determination of the average width and volume of the intermenbrane space of mitochondria

On the electron micrographs at x 70,000, the average width ($\bar{a}$) of the intermembrane space (IS) of mitochondria was measured. Only organelles of large diameter were considered, based on the assumption that they were cut according to a plane passing through their geometrical centre. The average volume of IS ($\bar{V}_{is}$) was estimated as difference of the volumes of two spheres of diameter $\bar{D}_{mit}$ and $\bar{D}_{mit} - 2\bar{a}$, respectively. Vv_{oc} was then recalculated from $\bar{V}_{mit}$ and $\bar{V}_{is}$; in this case Vv_{oc} displayed an underestimation of about 32%, which can be explained by the fact that Vv_{oc} measurement performed by «differential point counting» also included the thickness of the outer and inner mitochondrial membranes (Fig. 1).

Statistics

The data obtained from each rat were averaged per age group, and the SD of the mean was calculated. The statistical comparison of the data was performed by ANOVA followed by the Multiple Range Test of Duncan.

Results

Basal PREG production by dispersed ZF cells declined with advancing age (-27% and -39% at the 10th and 16th month), while PREG synthesis from 25-hydroxycholesterol by ZF mitochondria underwent a marked rise (2.2- and 2.7-fold at the 10th and 16th month) (Table 1).

Vv_{mit}, Vv_{ma} and Vv_{cs}, as well as Sv_{im} did not display significant age-dependent changes (Table 2). Conversely, at the 10th and 16th month of age, Vv_{oc} and Sv_{om} (either calculated by «differential point counting» or linear measurements) significantly rose (10-11% and 9-10% at the 10th month, and 15% and 16% at the 16th month) (Tables 2, 3).

$\bar{D}_{mit}$, $\bar{V}_{mit}$ and $\bar{S}_{mit}$ displayed an age-related decrease (-9%, -25% and -18%, respectively, at the 10th month, and -14%, -35%, and -25%, respectively, at the 16th month of age) (Table 3). The width of IS remained unchanged (averaging 12 nm), but $\bar{V}_{is}$ markedly decreased (-17% and -26% at the 10th and 16th month,

Table 1. Effect of ageing on PREG production by dispersed cells and isolated mitochondria of rat adrenal ZF (means±SD; n=8)

AGE (months)	4	10	16
Dispersed cells (pmol/10^6cells.h)	265.4±91.2	194.7±62.5*	160.8±56.4**
Isolated mitochondria (nmol/mg protein.h)	18.9±6.0	40.9±16.5**	50.7±19.3**

*: $p<0.05$ and **$p<0.01$ from 4-month-old rats.

Table 2. Age-dependent changes in the morphometric parameters of rat ZF mitochondria, as evaluated by «differential point counting» and «linear intersection counting» (means±SD; n=8).

AGE (months)	4	10	16
Vv_{mit}	0.330±0.058	0.326±0.063	0.331±0.060
Vv_{oc}	0.108±0.011	0.119±0.013*	0.124±0.013**
Vv_{ma}	0.440±0.038	0.427±0.044	0.427±0.032
Vv_{cs}	0.452±0.049	0.454±0.057	0.449±0.048
Sv_{om}	5.686±0.589	6.198±0.598*	6.580±0.619**
Sv_{im}	18.814±1.919	18.925±1.954	18.953±2.047

Vv_{mit}t: volume density of the mitochondrial compartment ($\mu m^3/\mu m^3$ of cell); Vv: volume density of the submitochondrial compartment indicated by the suffix ($\mu m^3/\mu m^3$ of mitochondria); Sv: surface density of the submitochondrial compartment indicated by the suffix ($\mu m^3/\mu m^3$ of mitochondria). Suffixes: cs, cristal space; im, inner (cristal) membrane; ma, matrix; oc, outer compartment; om, outer membrane. *: $p<005$, and **$p<0.01$ from 4-month-old rats.

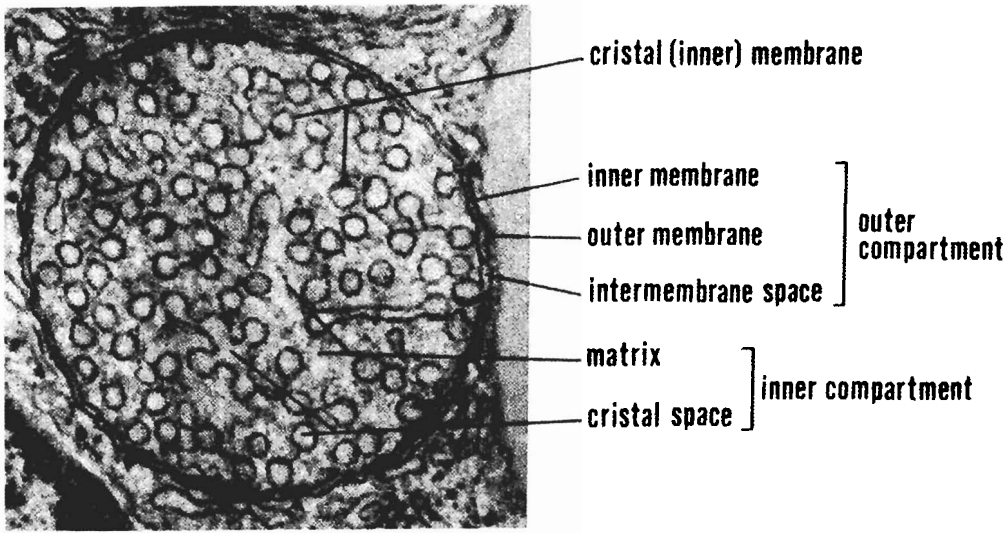

Fig. 1. Mitochondrion of the ZF of rat adrenal cortex, showing its significant compartments and structures. x 70,000

Table 3. Age-dependent changes in the morphometric parameters of rat ZF mitochondria, as evaluated by linear measurements (mean±SD; n=8).

AGE (months)	4	10	16
$\bar{D}_{mit}$	0.970±0.091	0.880±0.086*	0.839±0.079**
$\bar{V}_{mit}$	0.478±0.083	0.357±0.056**	0.309±0.058**
$\bar{S}_{mit}$	2.954±0.580	2.432±0.496*	2.210±0.435**
$\bar{a}_{is}$	0.012±0.001	0.012±0.001	0.012±0.001
$\bar{V}_{is}$	0.035±0.004	0.029±0.003**	0.026±0.003**
Nv_{mit}	0.690±0.074	0.913±0.096**	1.071±0.124**
Sv_{om}	6.181±0.609	6.811±0.659*	7.153±0.695**
Vv_{oc}	0.073±0.007	0.081±0.008*	0.084±0.008**

$\bar{D}_{mit}$: average diameter of mitochondria (μm); $\bar{V}_{mit}$: average volume of mitochondria (μm³); $\bar{S}_{mit}$: average surface area of the outer mitochondrial membrane (μm²); $\bar{a}_{is}$: average width of the intermembrane space (μm); $\bar{V}_{is}$: average volume of the intermembrane space (μm³); Nv_{mit}: numerical density of mitochondria (n/μm³ of cell). Other abbreviations as in Table 2. *p<0.05, and **p<0.01 from 4-month old rats.

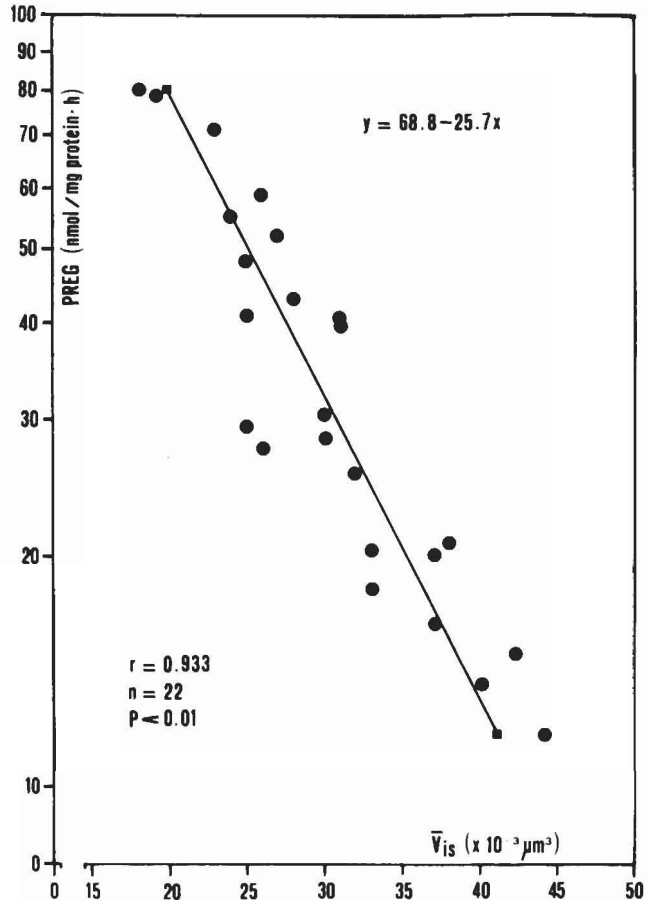

Fig. 2. Negative semilogarithmic correlation between the volume of the intermembrane space of rat ZF mitochondria and PREG production by isolated mitochondria. The regression line was obtained by the least square method, and the significance of the correlation was tested by the r coefficient of Pearson.

respectively) (Table 3). Nv_{mit} underwent a striking age-dependent rise (32% and 55% at the 10th and 16th month, respectively) (Table 3).

Discussion

According to previous studies (see Introduction), our present findings clearly show that the age-dependent decline in the secretory activity of rat ZF cells is caused by a lesion of their steroidogenic machinery located prior to the action of the cytochrome-$P450_{scc}$, inasmuch as the capacity of mitochondria to convert free cholesterol to PREG is markedly raised during ageing.

In interpreting this last finding, it may be taken into account that the conversion of cholesterol to PREG is a rather complex process involving the following three main steps: (i) transfer of free cholesterol to the outer mitochondrial membrane, a process requiring a cytoplasmic carrier named SCP_2 protein; (ii) translocation of cholesterol from the outer to the inner (cristal) mitochondrial membranes, where cytochrome-$P450_{scc}$ is located; and (iii) cleavage of cholesterol side-chain by cytochrome-$P450_{scc}$ (for review, see Jefcoate et al., 1992). On these grounds, many mechanisms can theoretically be assumed to underlie the age-dependent increase in mitochondrial conversion of cholesterol to PREG, which can be reasonably interpreted as a response to the prolonged exposure to high levels of circulating ACTH (see Introduction).

An age-related increase in the transfer of free cholesterol to mitochondria (1st step) can be excluded, since PREG production by dispersed intact ZF cells is decreased, due to the lowered availability of free cholesterol, which in turn is caused by the decline in the activity of neutral cholesteryl-esterase (Popplewell and Azhar, 1987).

It is well known that ACTH enhances the transcription of the genes of several enzymes of steroid synthesis, including cytochrome-$P450_{scc}$ (for review, see Miller, 1989; Simpson et al., 1990): thus an increased de novo synthesis of cytochrome-$P450_{scc}$ could occur during ageing (3rd step). Our stereological data seem to rule out this possibility. Evidence is available that the amount of enzymes of steroid synthesis (Δ^5-3ß-hydroxysteroid dehydrogenase and 11ß-hydroxylase) is tightly coupled with the surface area of the membrane in which they are inserted (Nussdorfer and Mazzocchi, 1983; Nussdorfer, 1986). However, the surface density of mitochondrial cristae does not display any age-related change. Furthermore, it must be noted that an increased amount of cytochrome-$P450_{scc}$ would be ineffective if the amount of substrate (i.e. free cholesterol) were not proportionally raised. Hence, the most probable mechanism involved in the age-dependent increase in mitochondrial production of PREG appears to be an enhanced translocation of cholesterol through the intermembrane space (2nd step).

The acqueous intermembrane space is a barrier to the

movement of non-polar sterols, and the involvement of steroidogenic labile and not labile proteins seems to be necessary to overcome it (Epstein et al., 1989). Among these proteins, a pivotal role is played by an endogenous ligand (called diazepam binding inhibitor, DBI), that binds to the mitochondrial benzodiazepine receptors (for review, see Krueger and Papadopoulos, 1992; Whitehouse, 1992). The possibility that the prolonged exposure to elevated ACTH concentrations enhances the expression of such proteins cannot be excluded; however, we want to recall that DBI level is not regulated by ACTH (Brown et al., 1992).

As mentioned in the Introduction, the possibility that physical changes in mitochondria can facilitate cholesterol translocation seems to be the most convincing one. Our stereological data show that the volume density of the outer mitochondrial compartment (Vv_{oc}) markedly increases with advancing age. This finding, which is in keeping with the results obtained by Boshier et al. (1990) in acutely ACTH-administered rats, indicates that per unit volume of mitochondria the volume of the acqueous barrier is increased, which should hamper rather than facilitate cholesterol translocation to the cytochrome-$P450_{scc}$. Caution, however, must be taken when interpreting relative stereological parameters. In fact, during ageing, the width of the intermembrane space remains unchanged, while the average volume of mitochondria steadily decreases, so that the average volume per single organelle of the intermembrane space significantly declines. This finding fits well with a facilitation of cholesterol translocation in single organelles. The highly significant semilogarithmic inverse correlation between PREG production by isolated mitochondria and average volume of mitochondrial intermembrane space (Fig. 2) lends support to this contention.

The decrease in the average volume of mitochondria is coupled with the increase in the number of these organelles, which explains why volume density of mitochondrial compartment (Vv_{mit}) remains unchanged during ageing. Previous investigations have shown that prolonged ACTH exposure induces a marked proliferation of mitochondria in rat ZF cells, mainly due to division of pre-existing organelles and which is obviously associated with the decrease in their average volume (Nussdorfer et al., 1974; Nussdorfer, 1986). It is reasonable to assume that this may also occur in aged rats, whose adrenocortical cells are exposed to high levels of circulating ACTH.

In conclusion, it seems legitimate to hypothesize that all the above-described age-related mitochondrial changes are ACTH-dependent compensatory responses enabling rat ZF cells to face their decreased secretion of steroids, due to the partial impairment of free-cholesterol supply. In light of the present coupled biochemical and stereological findings, the conclusion can also be drawn that, as far as rat adrenocortical mitochondria are concerned, «small» is «better», since of two mitochondrial populations of equal volume the one composed of smaller and more numerous organelles is the more efficient in the utilization of cholesterol in steroid synthesis.

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