AGING-LIKE ALTERATIONS OF SDH ACTIVITY IN PURKINJE CELL MITOCHONDRIA OF ADULT VITAMIN-E DEFICIENT RATS

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ABSTRACT

The ultrastructural features of perikaryal mitochondria positive to the copper ferrocyanide cytochemical reaction due to SDH activity were investigated in Purkinje cells of adult rats fed a vitamin E (α -tocopherol) deficient diet (AVED) for 11 months. The mitochondrial volume fraction (volume density: Vv), the number of organelles/ μm^3 of tissue (numeric density: Nv) and their average volume (V) were estimated by computer-assisted morphometry. The data obtained were compared with our previous results on 3, 12 and 24 month-old normally fed rats. In a comparison with age-matched controls, AVED animals showed significant decreases of the three morphometric parameters taken into account. These reductions were also observed in old, normally fed rats vs. the young and adult groups, but in AVED rats Vv and V decreased to a higher extent. In adult control animals, the percent of larger organelles ($0.32 \mu\text{m}^3 >$) decreases to less than 1%. Vitamin E deficiency resulted in a steeper reduction of this fraction of organelles, i.e. only 0.5% in the $0.24 - 0.32 \mu\text{m}^3$ size range accounted for the largest mitochondria in the AVED group. Taken together, these data document a significant impairment of mitochondrial efficiency in old and AVED rats. We interpret these findings to support that the underlying processes of aging and vitamin E deficiency may share common mechanisms. Considering the antioxidant action of α -tocopherol and the SDH role in cellular bioenergetics, inadequate protection from free radical attacks appears to represent an important determinant in the age-related decline of the mitochondrial metabolic competence.

INTRODUCTION

A growing body of experimental evidence is documenting that with advancing age cellular bioenergetic mechanisms undergo a progressive decay (1, 2). A major determinant of this impairment appears to be the age-related decline in the mitochondrial efficiency to provide adequate amounts of adenosinetriphosphate (ATP) in due time (3-5). Since mitochondrial membranes constitute primary targets for free radical attacks generated during the physiological oxidative phosphorylation (OXPHOS) process, with advancing age, these organelles are at high risk of serious damage particularly in postmitotic cells such as neurones (6, 7).

Several molecules are reported to counteract free radical attacks and, among these, vitamin E (α -tocopherol) is supported to interrupt the lipid peroxidation chain and to act as a biological antioxidant by stabilising the architecture of cellular membranes (8-10). Conceivably, experimental vitamin E deficiency results in consistent impairment of important membrane functions.

Succinic dehydrogenase (SDH), a key molecule of complex II of the OXPHOS process, is a secondary entrance of the electron transport chain. At variance with the other OXPHOS molecules, it is completely synthesised by the nuclear genome and then it is transported within the mitochondrion to be allocated at the inner membrane of the organelle. SDH is the only mitochondrial enzyme common both for electron and carbon fluxes, thus representing the unique molecule for cross control between cellular respiration and the Krebs cycle (11, 12). On the basis of these features, quantitative evaluations of SDH activity may provide reliable information on the mitochondrial capabilities for ATP production.

Continuing our previous studies on the bioenergetics of large cells in the aging nervous system (13, 14), we estimated SDH activity in cerebellar Purkinje cells of adult vitamin E-deficient rats (AVED) by means of computer-assisted cytochemistry. This study was undertaken to assess whether the absence from diet of such a protecting molecule results in an aging-like weakening of the mitochondrial metabolic competence.

RESULTS

The copper ferrocyanide cytochemical reaction constitutes a very specific staining procedure to evidence SDH activity. As documented by several authors including ourselves (13-19), the precipitates are due to the very rapid acceptance of electrons by ferricyanide which is reduced to ferrocyanide. In turn, this molecule is captured by copper at the site of enzyme activity. This sequence of chemical reactions occurs at the SDH location within the inner mitochondrial membrane and results in very discrete spots which are too small for reliable morphometric evaluations. In order to enlarge selectively these cytochemical deposits, uranyl acetate and lead citrate may be used as contrasting agents provided that staining time, concentration of the reagents and type of solvent are taken into account. The optimal balance among these determinants may lead to a consistent improvement of the fine localisation of the dark precipitates due to SDH activity. The electron

microscopic picture reported in Fig. 1, was obtained by short immersion (30 seconds) of the ultrathin sections in aqueous uranyl acetate followed by a longer immersion (5 minutes) in aqueous lead citrate. At present, this schedule appears to result in a satisfactory staining of our samples, however we are still investigating these steps in order to get an improved contrast.

Fig. 1. Electron microscopic picture of SDH-positive mitochondria in a Purkinje cell perikaryon of an adult, vitamin E-deficient rat. The dark precipitates are due to the reaction of the enzyme with copper ferricyanide. The background is markedly electron lucent since the cytochemical procedure is carried out on fresh, unfixed material. Bar: 0.5 μ m.

Figure 2 reports the results of Nv. AVED animals and the old group of rats showed almost the same value of Nv, which is significantly decreased vs. 3 and 12-month-old animals. No significant difference was observed between young and adult groups.

Fig. 2. Numeric density of SDH-positive mitochondria (Nv) in rat Purkinje cell perikarya. *: $p < 0.001$ vs. the adult group.

The results of V are shown in Fig. 3. AVED rats showed a significant decrease of this parameter vs. the three groups of age taken into account. V decreases significantly in adult as well as old animals when compared with the young group of rats. No significant difference was observed between 12 and 24 months of age.

Fig. 3. Average volume of SDH-positive mitochondria (V) in rat Purkinje cell perikarya. *: $p < 0.05$ vs. the adult and old groups; •: $p < 0.001$ vs. the age groups.

In the AVED rats the volume fraction (Vv) occupied by the SDH-positive mitochondria (Fig. 4) was significantly lower than in the other groups of age taken into account. Young and adult rats showed almost the same value of this parameter, which appeared significantly decreased when compared with the old group of animals.

Fig. 4. Volume density (or volume fraction) of SDH-positive mitochondria (Vv) in rat Purkinje cell perikarya. •: $p < 0.001$ vs. the age groups.

Fig. 5. Size (V) distribution of SDH-positive mitochondria in rat Purkinje cell perikarya. Young and old animals show a high percentage of large organelles (0.32 μm^3). In adult animals, large mitochondria account for less than 1% (small arrow). This alteration is more evident in AVED rats: the same percentage of largest organelles is found in the 0.24-0.32 μm^3 size range (big arrow).

A size (V) distribution of the population of SDH-positive mitochondria is reported in Fig. 5. Young and old animals show the same pattern which is characterised by a consistent percentage (13 and 11%, respectively) of organelles of larger volumes (0.32 μm^3). In the adult animals, this percentage decreases to less than 1%, (small arrow). In the AVED group, more than 72% of the SDH-positive organelles are of small size (0.08 – 0.16 μm^3); the 0.32 μm^3 size fraction disappears, but the amount of largest mitochondria is documented by the 0.5% found in the 0.24-0.32 μm^3 size range (big arrow).

DISCUSSION

Vitamin E deficiency resulted in a significant decrease of the numeric density as well as of the average size of SDH-positive mitochondria in Purkinje cell perikarya of adult rats: as a final outcome, the volume fraction occupied by the organelles was also significantly reduced. The mitochondrial size distribution supports that the above alterations are due to the consistent decrease of the percent of larger organelles. Similar changes were found in old rats, thus vitamin E deficiency appears to cause an aging-like decay of SDH activity in Purkinje cell mitochondria.

Reasonable interpretations to explain the alterations found by us in AVED rats must take into account both the widely claimed antioxidant action of α -tocopherol and the functional role played by SDH in the OXPHOS process.

Besides acting as a free radical scavenger, Vitamin E is reliably documented to stabilise cellular membranes. In details, it has been demonstrated that the presence in cell culture media of different concentrations of α -tocopherol is able to modulate the architecture of cellular membranes by controlling their unsaturated phospholipid and cholesterol profiles (9, 20). Within plasma membranes, vitamin E appears to be not homo-

geneously distributed, on the contrary, it is reported to be more abundant at the very fluid zones, thus α -tocopherol is associated with the most unsaturated fatty acyl chains where more protection is needed (21). Converging evidence suggests that an adequate amount of vitamin E within membranes may decrease the number of sites available for free radical attacks by establishing stereospecific interactions with the surrounding lipid moieties (22). With specific reference to mitochondria, investigations on liver and muscle have reported that the inner membrane of organelles from vitamin E-deficient animals is particularly sensitive to lipid peroxidation which is able to cause disorder of the phospholipid movements in the vertical plane of the membrane. As a consequence, the capacity to maintain tight electrical and ionic coupling is impaired and the mitochondrial membrane functions markedly decline (23).

In addition to the very specific features described in the introduction, the choice of SDH preferential cytochemistry to estimate mitochondrial efficiency deserves further comments. First of all, succinate (as well as glutamate – malate) is reported to support maximum rates of respiration: for nerve cells, which are characterised by frequent bursts of activity, this represents an almost physiological condition requiring adequate ATP amounts. Conceivably, the mitochondrial capacity for ATP provision in neurones is continuously challenged by these normal, ongoing cellular performances. Thus, the evaluation of SDH activity may constitute a reliable metabolic marker which also appears to be proportional to the energy production needed by adult nerve cells (24). Taking into account the specific location of the components of the respiratory chain, it must be remarked that SDH molecule is an integral component of the inner mitochondrial membrane therefore its activity is significantly influenced by the physico-chemical conditions (e.g. fluidity) of the membrane as well as by membrane phospholipid-protein interactions. As a consequence, the lipid environment may change SDH configuration from the inactivated to the activated state and this may affect ATP production (12). All these features lend further support not only to the central role of SDH in cellular bioenergetics, but also to its sensitivity to several factors.

As reported by many research reports, mitochondria are very plastic organelles undergoing different turnover cycles according to the type of cells as well as cellular location (25, 26). These physiological processes aim at adapting mitochondria to the energy demands and involve the structural remodelling of each organelle. Conceivably, assessments of the dynamic rearrangements of the whole mitochondrial population may be estimated by considering N_v , V and V_v all together per experimental group of animals. In this context, our present findings support that both in old and AVED animals, the mitochondrial capacities for morphofunctional plasticity are significantly impaired. The causative events responsible of these alterations

appear to be shared by aging and vitamin E deficiency and may be singled out as the ubiquitous deterioration due to uncontrolled oxidative processes markedly occurring in both these conditions. An increased production of oxidants has been documented by the increased activity of phospholipase A₂ at the inner membranes of liver mitochondria of old (27) and adult vitamin E-deficient rats (28), thus strengthening the concept of the high risk of damage of these organelles to free radical attacks. With specific reference to SDH, the above assumption is supported by the wealth of current experimental data confirming that membrane functions are seriously compromised both in aging and vitamin E deficiency and this, in turn, may affect SDH specificity (29, 30) as well as modify the enzyme's molecular structure (31).

Oxidants escaping control mechanisms may cause alterations not only at mitochondrial membranes, but also at mitochondrial DNA (mtDNA) with the consequence of a significant proliferation of defective organelles. Recent reports on mitochondrial genetics lend support to this assumption and prompted to state that the mitochondrial population of old postmitotic cells is composed of a mosaic of organelles with different metabolic potentials (32, 33). Since mtDNA is attached to the inner mitochondrial membrane, this macromolecule may be reasonably assumed to represent a preferential candidate for free radical damage. However, each organelle denotes a discrete functional unit of the cellular bioenergetic machinery, thus any causal determinant contributing to preserve the mitochondrial structural integrity may play a critical role in the organelle's functional performances and supports the rationale of correlating mitochondrial morphological features with its metabolic competence. In this context, the morphometric quantitation of SDH-positive organelles by the copper ferrocyanide cytochemical procedure appears to provide information on the mitochondrial fraction supported to be metabolically active (24, 34, 35). Moreover, each parameter taken into account by our investigation reports on single discrete aspects of the mitochondrial metabolic competence and may enable to visualise very specific differences. Namely, the percent distribution shown in Fig. 5 documents that in adult control rats, mitochondria with an increased size constitute a very small percentage of $0.32 > \mu\text{m}^3$ organelles (small arrow), while in AVED rats almost the same percentage accounts for the largest mitochondria in the $0.24\text{--}0.32 \mu\text{m}^3$ size range (big arrow). Despite vitamin E-deficiency in young laboratory animals has been considered a reliable model of precocious aging, we found that in the old normally fed rats $0.32 > \mu\text{m}^3$ organelles account for more than 10%, thus some differences are clearly apparent between aging and a vitamin E-deficient dietary regimen, at least as regards SDH-positive mitochondria. Considering the ordered sequence of events occurring in mitochondrial genesis and renewal, the largest mitochondria represent the fraction of organelles which are going to divide in order

to increase the mitochondrial numeric density and, in turn, the cellular metabolic capacities to provide ATP, thus our findings may be interpreted as different morphofunctional patterns featuring the mitochondrial structural dynamics. In agreement with this rationale, the steeper decrease of the percentage of smaller mitochondria in AVED rats vs. adult controls supports the idea that lack of protection from free radical attacks results in an accelerated mitochondrial deterioration which, in addition to reduce the overall number of organelles, is also responsible of an impaired mitochondrial plasticity favoured by the increased oxidative stress conditions. In physiological aging and also in young animals, the mitochondrial potential for adaptive response appears to be still active as supported by the presence of the consistent percentages of very large organelles. In vitamin E deficiency, the absence of this compensatory response to such an unfavourable condition suggests that long lasting increased oxidative stress must be considered a very dangerous situation promoting a quasi-pathological condition which weakens the cell adaptive potential and constitutes a serious threat for its longevity.

As hippocampal and cortical pyramidal cells, Purkinje cells are very large neurones characterised by a high biosynthetic energy demand due to their long axonal projections and big soma size. These features make them very sensitive to aging. Our present findings confirm and extend this concept previously supported by SDH investigations we carried out on hippocampal CA1 pyramidal cells (13). Moreover, the data on AVED rats support that the mitochondria of these large neurones are markedly vulnerable to uncontrolled oxidation-reduction reactions within the cells. In turn, the present findings stress the importance of dietary antioxidants in preventing free radical-mediated deteriorations occurring at mitochondrial membranes.

EXPERIMENTAL PROCEDURES

We used female Wistar rats of our own breed. From weaning, five 1-month-old animals were fed a vitamin E deficient diet according to our previous experimental paradigm (34). These animals and their controls, i.e. normally fed littermates, were sacrificed at 12 months of age. As reported in our previous papers (13, 14), SDH activity was preferentially evidenced by means of the copper ferrocyanide cytochemical reaction at discrete sites of the inner mitochondrial membrane (Fig. 1). Briefly, the three anterior segments of the right cerebellar lobule were chosen as anatomical model for our study. Five to eight slices were obtained from the fresh tissue by means of a McIlwain tissue chopper. These samples and those from controls, respectively, were then immersed for 45 minutes at 37°C in substrate or substrate-free incubation media (see the detailed composition refs. 13, 14, 35). Postfixation, dehydration and embedding were carried out according to conventional electron microscopic procedures.

Morphometry. The ²equal opportunity rule² was applied on trimming our samples, i.e. the first Purkinje cells observed under the light microscope were chosen to prepare sections to be analysed at the electron microscope. At the fixed periodicity of 10 slices, we measured the SDH positive mitochondria according to our previous studies (13, 14 and 35). An overall surface of 8,400 mm² of perikaryal area was sampled per experimental group. The number of mitochondria/mm³ of cytoplasm (numeric density: Nv), the average mitochondrial volume (V) and the total mitochondrial volume/mm³ of cytoplasm (volume density: Vv) were the parameters semiautomatically measured by computer-assisted morphometric methods on SDH-positive organelles (13, 14). The data referred to the age of the animals, i.e. 3 (young), 12 (adult) and 24 (old) months of age, are taken from our previous paper (35). Statistical comparisons were performed by one way analysis of variance (ANOVA) and the Student-Newman-Keuls' test.

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ABBREVIATIONS

ATP = adenosinetriphosphate; ANOVA = analysis of variance; AVED = adult-vitamin E-deficient; μ m = micron; Nv = mitochondrial numeric density; OXPHOS = oxidative phosphorylation; SDH = Succinic dehydrogenase; V = mitochondrial average volume; Vv = mitochondrial volume density or volume fraction.

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