FEBS Letters 380 (1996) 176–178 FEBS 16651

Inhibitor sensitivity of respiratory complex I in human platelets: a possible biomarker of ageing

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Received 4 December 1995; revised version received 29 December 1995

Abstract NADH-Coenzyme Q reductase was assayed in platelet mitochondrial membranes obtained from 19 pools of two venous blood samples from female young (19–30 years) individuals and 18 pools from aged ones (66–107 years). The enzyme activities were not significantly changed in the two groups, but a decrease of sensitivity to the specific inhibitor, rotenone, occurred in a substantial number of aged individuals. The results are in agreement with the predictions of the mitochondrial theory of ageing and may be used to develop a sensitive biomarker of the ageing process.

Key words: Ageing; Platelet mitochondrion; Complex I; Rotenone sensitivity

1. Introduction

According to the mitochondrial theory of ageing [1], accumulation of somatic mutations in mitochondrial DNA (mtDNA), due to continuous attack by oxygen-derived radicals and other toxic species, is a key factor in determining the decline in cell performance that characterizes senescence.

Mitochondrial DNA encodes for 13 hydrophobic polypeptide chains of the four enzymic complexes of the mitochondrial inner membrane involved in oxidative phosphorylation [2]. Since 7 out of the 13 chains are subunits of Complex I (NADH-Coenzyme Q reductase) of the respiratory chain, it is predicted that the highest frequency of mutations would affect this enzyme. A decreased activity of Complex I was indeed found in experimental animal tissues and muscle biopsies from aged individuals [3–5].

Recently, the medical interest in Complex I has increased in view of its involvement in a number of genetic and acquired degenerative diseases [6]; particularly interesting is Leber's hereditary optic neuropathy (LHON), a maternally inherited disease associated to mtDNA point mutations: the most frequent form of LHON is associated to increased resistance to rotenone, a classical inhibitor of the Complex I that binds to the hydrophobic sector [7].

Since the common 5 kb deletion, and others as well, described to increase in ageing, encompass a region including subunits of Complex I [8], we have investigated the specific activity and turnover of NADH-Coenzyme Q reductase and its rotenone sensitivity, in an attempt to find a marker of the bioenergetic lesions occurring in senescence. For this pilot study we have employed mitochondrial membranes from human platelets, as these cells are of easy sampling and do not involve invasive procedures.

2. Materials and methods

Platelets were obtained from venous blood samples (60 ml each), pooled with two by two, from 38 female young (19–30 years) individuals (19 pools) and 36 aged ones (66–107 years; 18 pools) selected by the lack of systemic and haematic diseases. Since it has been easier to recruit old female individuals from the hospitalized group of aged subjects, we decided to consider only females to keep the group homogeneous. Blood samples of each pool were from age-matched individuals.

Platelets were purified following the method of Blass [9] with the following modifications; erythrocytes were sedimented in 70000 dextrane, 0.9% NaCl at room temperature for 30 min. The upper phase was centrifuged ($3{,}000 \times g \cdot 3$ min) and an aliquot of the platelet rich plasma supernatant was tested by a Cell Counter to determine the mean platelet volume; the remainder was centrifuged at $4{,}000 \times g$ for 25 min then washed in 20 mM sodium phosphate, 0.12 M NaCl, pH 7.4 ($5{,}000 \times g \cdot 10$ min). In order to prepare membrane fragments including mitochondrial particles, the pellet of platelets was treated as described by Degli Esposti et al. [7] with the following modifications: the pellet was subjected to osmotic shock to remove residual erythrocytes, washed ($15{,}000 \times g \cdot 10$ min) in 150 mM KCl, 50 mM Tris-HCl, 1 mM EDTA, pH 7.4, and mildly sonicated five times at 10-s periods (150 Hz) with 50-s intervals.

The platelet membrane fragments, that include the mitochondrial ones, were diluted 1:1 in sucrose 0.25 M, Tris 30 mM, EDTA 1 mM, pH 7.7, then separated from the heavier cell debris by centrifugation ($33{,}000 \times g \cdot 10$ min); the supernatant was then ultracentrifuged ($100{,}000 \times g \cdot 40$ min) and the pellet was suspended in the same buffer. The membrane preparation contained ruptured mitochondrial fragments lacking permeability barriers, as ascertained by the presence of both NADH oxidation and cytochrome oxidase activities. Protein content was assayed according to Lowry et al. [10].

Complex I activity (NADH-CoQ reductase, EC 1.6.99.3) was assayed utilizing an analog of the natural acceptor Coenzyme Q, decylubiquinone (DB, from Sigma, St. Louis, MO) [11]. The assay was performed [12] at 32°C in 50 mM K-phosphate, 10 mM KCN, 1 mM EDTA, pH 7.6, adding 75 μM NADH, 30 μM DB, and 50 μg/ml of protein, in a Sigma ZWS 2 dual wavelength spectrophotometer using the wavelength couple 340 minus 380 nm and an extinction coefficient of 5.5 $mM^{-1} \cdot cm^{-1}$.

Sensitivity to rotenone was assayed measuring specific activity after 10 min preincubation with increasing concentrations of inhibitor. The I_{50}, i.e. the inhibitor concentration eliciting half-inhibition, was taken as an indication of rotenone sensitivity.

NADH-ferricyanide reductase activity was used to estimate the content of active Complex I in the membrane using 2 mM K-ferricyanide, considering half of the turnover maximum ($8 \cdot 10^5$ min^{-1}) since the concentration of ferricyanide was approximately equal to the K_m [13]. K-ferricyanide is an hydrophilic acceptor and reacts with FMN enzyme-bound redox centre of Complex I; NADH-ferricyanide reductase activity was assayed essentially as described by Yagi [14] at 30°C, in

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SSDI 0014-5793(96)00037-3

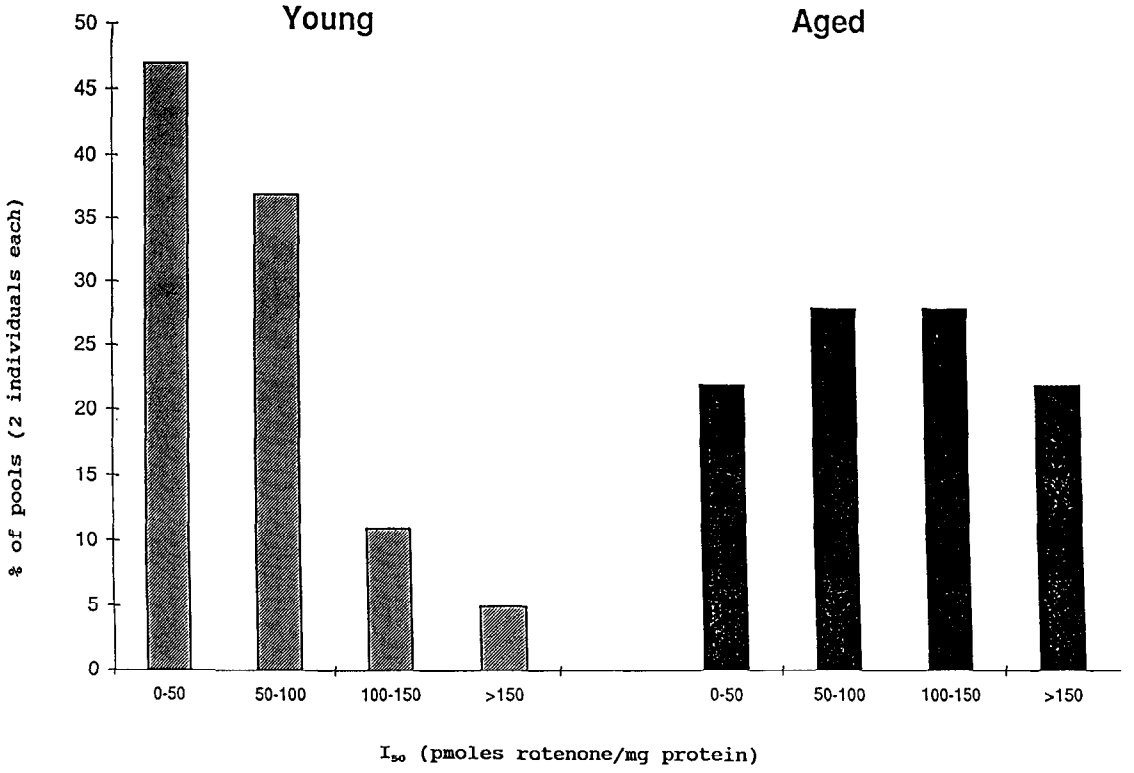

Fig. 1. Percent distribution in different classes of rotenone concentration half- inhibiting Complex I activity (I_{50}) in platelet membranes from young and aged individuals.

the same buffer used for NADH-DB reductase, with 2 mM K-ferricyanide, 150 μM NADH and 14 μg/ml of mitochondrial protein, in a Sigma ZWS 2 dual wavelength spectrophotometer using the wavelength couple 420 minus 500 nm and an extinction coefficient of 1 $mM^{-1} \cdot cm^{-1}$.

The turnover number of NADH-Coenzyme Q reductase, expressing the maximal efficiency of enzymatic activity, was calculated by the ratio NADH-DB activity:enzyme concentration.

Data are presented as means ±standard deviation. The significance of differences was evaluated by the unpaired t-test and accepted when $P < 0.05$.

3. Results

The mean platelet volume (fL), determined in 14 and 13 pools respectively from young and aged individuals, was slightly but significantly lower in the aged population (9.4 ± 0.2 vs. 8.8 ± 0.4, $P < 0.05$).

Table 1 shows no difference in the specific activities of both groups and a slight decrease in the turnover of Complex I in aged individuals.

The overall sensitivity to the specific inhibitor, rotenone, added in excess, was similar in both groups (71.8% vs. 76.1%).

It is worth noting, however, that the mean value of the I_{50} for rotenone inhibition was significantly higher in the aged group.

Fig. 1 reporting the percent distribution in different classes of I_{50} values of Complex I activity in platelet membranes from young and aged individuals, exhibits a dramatic shift to higher I_{50} classes in the old population: for example, in the aged group I_{50} values were comprised between 50 and 150 in 60% of the cases and between 0 and 50 in 22%, while almost 50% of the young showed I_{50} values between 0 and 50.

Table 1
Biochemical parameters in platelet membranes from young and aged female individuals

	Young (n = 19)	Aged (n = 18)
Complex I spec. activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)	4.6 ± 1.6	4.5 ± 0.9
Complex I turnover (s^{-1})	9.8 ± 4.6	7.4 ± 2.1
% rotenone sensitivity	76.1 ± 9.9	71.8 ± 10.0
I_{50} of rotenone ($pmol \cdot mg^{-1}$)	64.8 ± 44.8	126.1 ± 106.2*

Data refer to 19 platelet pools from young individuals and 18 pools from aged ones.
*$P < 0.03$
n = number of pools (2 individuals each)

4. Discussion

The finding of decreased rotenone sensitivity in ageing is compatible with the 'mitochondrial' theory. The postulated increase of somatic mutations of mtDNA will affect mostly those enzymes for whose subunits more genes are present. The hydrophobic subunits of Complex I, encoded by mtDNA, which are responsible among other functions for the sensitivity to rotenone and other inhibitors [15], are essential to the enzymatic function mainly for the mechanism of Coenzyme Q binding and energy conservation through H^+ translocation [16]; it can therefore be hypothesized that their alteration leads to decreased energy conservation more strongly than to decreased electron transfer. Mutations affecting energy conservation in Complex I may not be lethal for the cells and may be compatible with their replication if the other complexes still function,

whereas a loss of electron transfer in Complex I would lead to loss of activity of the whole respiratory chain with NAD-linked substrates.

The finding of a consistent mitochondrial alteration in platelets is somewhat surprising, as only postmitotic cells have been considered prone to accumulate mtDNA mutations [17]; in rapidly dividing cells, or in cells deriving from differentiation and then removed, it is expected that age-related changes may not accumulate to significant extent, because the defective cells would be 'washed out' by natural selction. It is considered however that these cells, which rely upon glycolysis as well as respiration, may develop mitochondrial changes still compatible with the cell's energetic function, particularly if the changes are not too severe. The choice of a kinetic parameter, as rotenone sensitivity, was dictated by the type of cell selected for this study. Platelets are relatively rich in mitochondria, but are known to have probably one copy only of mtDNA per mitochondrion [18]: thus even if age-related mutations preferentially lead to a heteroplasmic state in the cell [19], each mitochondrion, where a significant mutation occurs, would become energetically defective. The variability in rotenone sensitivity observed in the aged is expected, since not all mutations and/or deletions are expected to alter subunits involved in a single parameter as rotenone sensitivity. It is, however, noteworthy that the changes are observed in a significantly high number of individuals.

Although the type of lesion and its variability are in accordance with the expectancies of the mitochondrial theory of aging, we cannot exclude other possible reasons for the changes observed. Rotenone is a very hydrophobic molecule that acts through the lipid phase of the membrane: for this reason any change of lipid composition and fluidity of the inner mitochondrial membrane could affect its binding properties. It is of interest to point out that changes in the relative content of lipid classes were observed in senescent rats [20] and in platelets from old humans as well [21].

As this study was performed on a still limited number of subjects, it is at present impossible to directly relate the age of examined individuals to their sensitivity to rotenone. However, this pilot study is encouraging for the development of a biomarker of ageing: the use of rotenone sensitivity can be developed as a diagnostic and prognostic test for both normal ageing and development of age-associated diseases: the finding [22] that mtDNA 'common' deletion is higher in aged heart patients than in normal subjects of corresponding age encourages the search for a noninvasive test to predict the propensity to develop chronic diseases of ageing.

Acknowledgements: This study was supported by the CNR Target Project on Ageing, Code No. 951613, Rome. We are indebted to Prof. H. Baum and to Prof. G. Nubile for their useful advice during the course of this work. The competent assistance of M. Menigatti (student in the Medical School) is gratefully acknowledged. Control blood samples were kindly given by students of the Medical School.

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