Increased transcription of mitochondrial genes for Complex I in human platelets during ageing

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Received 12 August 2003; revised 29 August 2003; accepted 23 December 2003

First published online 6 January 2004

Edited by Vladimir Skulachev

Abstract We studied the effect of ageing on the mRNA levels of mitochondria-encoded polypeptides in human platelets. We used quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate the expression of selected cytochrome *c* oxidase (COX) genes (subunits I and III) and Complex I genes (subunits reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (ND)1 and ND5) in platelets from young and aged healthy subjects. Northern blot analysis confirmed the PCR results. COX I expression is higher than that of COX III in both young and aged platelets. A significant increase of transcripts for Complex I was found during ageing. On the contrary, the mRNA levels of the two COX subunits did not significantly vary during ageing.

Key words: Mitochondrial mRNA; Ageing; Oxidative phosphorylation system; Gene expression

1. Introduction

Ageing is a multifactorial phenomenon involving various changes at biochemical and morphological levels and is characterized by time-dependent decline in physiological functions. In particular ageing is associated with energy deficit and increased production of reactive oxygen species (ROS). Mitochondria are the major source and at the same time are targets of ROS [1]; this 'vicious cycle' [2] leads to an accumulation of damages to several molecules including the mitochondrial DNA (mtDNA) [3]. mtDNA is a circular genome of 16.5 kb encoding 13 polypeptides involved in oxidative phosphorylation (OXPHOS), two rRNAs (12S and 18S) and 22 tRNAs and is particularly susceptible to mutations. In addition, a variety of chronic degenerative diseases that involve brain, heart, muscle, kidney and endocrine glands have been shown to be connected to some mtDNA mutations [4,5].

Mitochondrial OXPHOS plays a fundamental role in energy production; it is located in the inner membrane and consists of five major multi-protein complexes. The majority of the subunits of the complexes are encoded by the nuclear genome with Complex II entirely encoded by nucleus. Complex I is the largest and the most unexplored one although it represents the key enzyme of the mitochondrial electron chain. Furthermore, since seven out of the 13 polypeptides encoded by mtDNA belong to Complex I (reduced nicotinamide adenine dinucleotide (NADH)-coenzyme Q reductase), it is expected that this enzyme should be mostly affected by ageing [6]. Progressive age-related OXPHOS impairment has been described in lymphocytes [7], muscle cells [8] and cardiomyocytes [9]. In particular a decrease of the enzymatic activity of Complexes I, II and IV in human skeletal muscle and an altered polypeptide pattern in cytochrome *c* oxidase (COX) have been observed [10].

The expression of mitochondrial genes in human cells has yet to be defined. Most studies on the effect of ageing on the mitochondrial transcription machinery refer to a non-human cell model [11–13]. Moreover, in most studies, the analysis was semiquantitative, such as Northern blotting.

Blood platelets have been recently proposed as biomarkers of mitochondrial function and ageing-related diseases [14–16] since they possess mitochondria and may be easily collected by non-invasive procedures. The rationale to employ platelets as a biomarker of mitochondrial lesions [17] rests on the assumption that alterations occurring in senescence and age-related diseases may be present in all cells, and that platelets may signal generalized bioenergetic deficiencies [18]. Platelet energy production derives mainly from glycolysis [17] and mitochondria are poorly involved in bioenergetic production. It is therefore expected that platelets with damaged mitochondria should not be removed by selective pressure, thus allowing their biochemical investigation.

The aim of this study was to quantify the mRNA levels of mitochondria-encoded polypeptides in human young and aged platelets. We investigated COX (subunits I and III) and Complex I subunits (NADH dehydrogenase (ND)1 and ND5) expression in platelets from young and aged subjects, using real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Healthy subjects

Fourteen young healthy subjects (median age 23 years, range 21–29)

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Abbreviations: mtDNA, mitochondrial DNA; ROS, reactive oxygen species; OXPHOS, oxidative phosphorylation; COX, cytochrome *c* oxidase; ND, reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

0014-5793/04/$30.00
doi:10.1016/S0014-5793(03)01520-5

Table 1
Primer sequences used for real-time RT-PCR analysis according to the Cambridge sequence

	COX I	COX III
FW primer	6247-CTG CTA TAG TGG AGG CCG GA-6266	9371-CCA ATG ATG GCG CGA TG-938
RW primer	6319-GGG TGG GAG TAG TTC CCT GC-6300	9438-CTT TTT GGA CAG GTG GTG TGT G-9417
Probe	6268-CAG GAA CAG GTT GAA CAG TCC ACC CTC CC-6296	9389-AAC ACG AGA AAG CAC ATA CCA AGG CCA-9415
	ND1	ND5
FW primer	3458-ACG CCA TAA AAC TCT TCA CCA AAG-3481	13278-AGT TAC AAT CGG CAT CAA CCA A-13299
RW primer	3461-TAG TAG AAG AGC GAT GGT GAG AGC TA-3536	13368-CCC GGA GCA CAT AAA TAG TAT GG-13346
Probe	3506-CCA TCA CCC TCT ACA TCA CCG CCC-3529	13311-ATT CCT GCA CAT GTG TAC CCA CGC CTT-13337

and nine aged healthy individuals (median age 70 years, range 69–82) were selected by Operative Unit of Immunohematology and Transfusion, S. Orsola-Malpighi Polyclinic, Bologna Hospital after informed consent.

In another set of experiments, we selected a second group of healthy subjects (three young, median age 23 years, range 21–29; five aged, median age 70 years, range 69–82). The RNAs extracted from the platelets of this group were pooled into two different samples (young and aged one). Northern blot analysis and quantitative RT-PCR have been applied to both pools (young and aged).

2.2. Platelet isolation

Platelets were obtained from 35 ml of whole blood as described by D'Aurelio et al. [18]. Blood was centrifuged at $150\times g$ for 15 min. To avoid leukocyte contamination, only the upper 8/10 of the platelet-rich plasma (PRP) was collected. PRP was diluted 1:1 with a buffer saline plus 5% sodium citrate and then centrifuged again at $1000\times g$ for 10 min. Erythrocyte contamination has been avoided by ammonium sulfate (1%) lysis. Light microscopy examination showed that the isolated platelets contained no detectable erythrocytes and less than 0.2% leukocyte contamination.

2.3. RNA extraction

Total RNA was isolated from platelets with Trizol reagent (Gibco BRL-Life Technologies) according to Chomczynski [19]. DNase treatment was performed to remove any trace of mtDNA (RNeasy Mini Kit, Qiagen). cDNAs were prepared from 1 μg RNA template in 50 μl reaction mixtures as previously described [20].

2.4. Real-time RT-PCR

The PCR primers and TaqMan probes to amplify and detect ND1, ND5, COX I and COX III genes were designed using the Primer Express software version 1.0 (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA) as shown in Table 1 and were based on GenBank nucleotide sequences. Quantitative RT-PCR has been performed as previously described [20]. The probes were purchased labeled with 6-carboxy-fluorescein (FAM) as the reporter dye, and with 6-carboxy-tetramethyl-rhodamine (TAMRE) as the quencher of fluorescence (Perkin-Elmer/Applied Biosystems). In order to minimize variability in the results due to differences in the RT efficiency and/or RNA integrity among the unknown samples, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was also tested. The TaqMan-GAPDH control reagents (Perkin-Elmer/Applied Biosystems) were used to amplify and detect GAPDH. Quantitation of each of the five genes was performed using standard curves established from plasmids containing their specific sequences. The real-time PCR was performed on an ABI PRISM 7700 sequence detector, using the ABI PRISM 7700 sequence detector software 1.6 (Perkin-Elmer/Applied Biosystems). All the reactions for samples or standard were run in triplicate. For quantification, expression of each gene was normalized by comparison with GAPDH expression. Normalized levels of unknown samples were calculated as the ratios between the investigated genes (ND1, ND5, COX I, and COX III) and GAPDH.

2.5. Northern blot

5 μg of RNA from the pools of young and aged individuals were resuspended in RNA loading buffer (50% formamide, 2.2 M formaldehyde and 1× electrophoresis buffer) and electrophoresed through a 1% agarose gel with 2 mM 4-morpholinepropanesulfonic acid, 0.5 mM sodium acetate and 0.1 mM ethylenediamine tetraacetic acid (EDTA) as the electrophoresis buffer. RNAs were transferred from the gel onto a nylon membrane and ultraviolet (UV) cross-linked for 1 min. The membrane was prehybridized at 42°C in prehybridization mix (50% ultrapure formamide, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate and 200 μg/ml sheared salmon sperm DNA) for over 4 h.

The cDNA probes used in this study were obtained as follows. Random primers for cDNAs were prepared from isolated mRNA by Moloney murine leukemia virus reverse transcriptase (Promega, Milan, Italy) after PCR amplification by thermal cycler. An aliquot of total RNA derived from the same samples of platelets of young and aged individuals was quantified spectrophotometrically. Levels of RNA bound to the Northern blot filter were assessed by gel electrophoresis analysis.

3. Results

Using real-time RT-PCR, we quantitated the COX I, COX III, ND1 and ND5 mRNAs in each sample, using for normalization the housekeeping gene GAPDH. Data are shown in Fig. 1. A standard curve was then constructed plotting the cycle threshold (Ct) against the known copy number of each standard sample. There was a linear decrease of the Ct in proportion to the log of the starting copy number with a correlation coefficient ranging from 0.97 to 1.00 in the five genes. The signal from each sample was normalized to the signal obtained from the same RNA non-retro-transcribed to eliminate any error due to amplification of RNA templates.

The COX III, ND1 and ND5/GAPDH ratios are quite

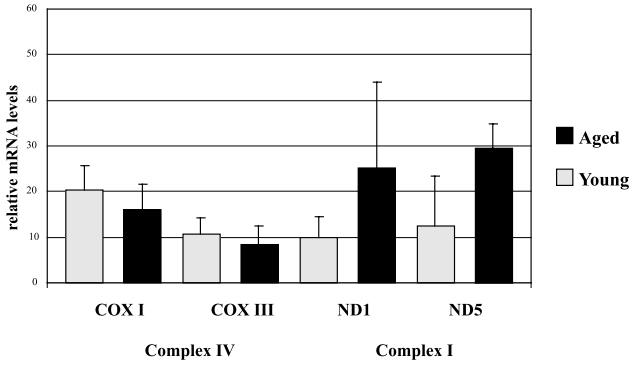

Fig. 1. Quantitative RT-PCR TaqMan analysis of mtDNA gene expression (Complex I: ND1 and ND5 subunits; Complex IV: COX I and COX III subunits) normalized to GAPDH housekeeping gene in young and aged individuals. The data are shown as mean ± standard deviation. Relative mRNA levels are the amounts of mRNA for mitochondrial subunits over mRNA for GAPDH. COX I/COX III (young) $P = 0.0001$; COX I/COX III (aged) $P = 0.0065$; ND1 (young/aged) $P = 0.049$; ND5 (young/aged) $P = 0.0049$.

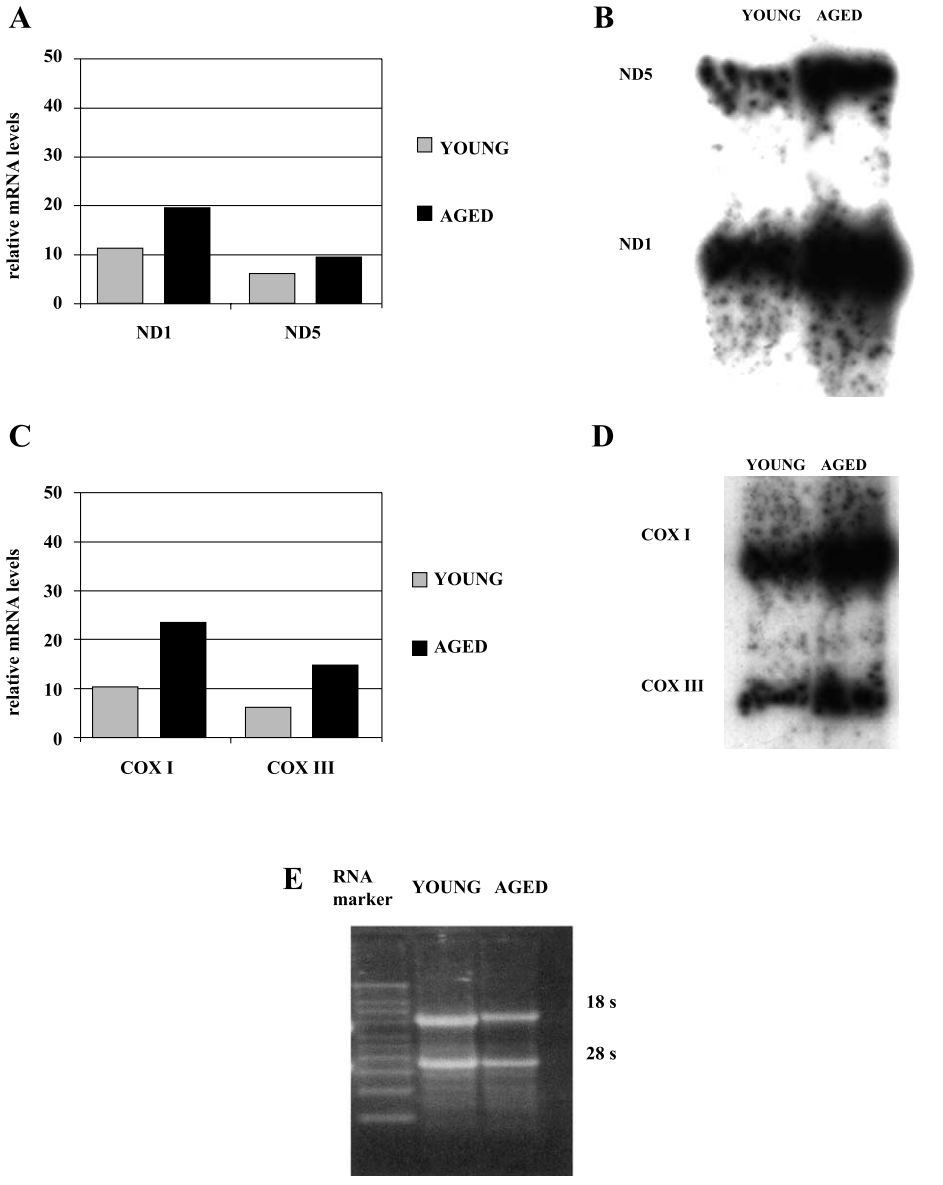

Fig. 2. Comparison of real-time RT-PCR TaqMan analysis and Northern blot analysis of mitochondrial gene expression from two pools of mtRNA respectively from young and aged individuals. A: Real-time PCR of ND1 and ND5. B: Northern blot of ND1 and ND5. C: Real-time PCR of COX I and COX III. D: Northern blot of COX I and COX III. Note the low values for COX I and COX III in the young in this experiment (C). For the Northern blot analysis, total RNA (10 μg) from each pooled young and aged group was resolved by gel electrophoresis in a 1% agarose gel (E) to monitor the amount of RNA in each lane (appreciatively equal amount of 28S and 18S RNA, indicating equal loading in each lane). For RT-PCR TaqMan data see the legend of Fig. 1.

similar in platelets from young individuals (Fig. 1). By contrast the COX I/GAPDH ratio was about twice in comparison with the other genes. The same genes show a different pattern of expression in platelets from the aged subjects. COX I/GAPDH and COX III/GAPDH ratios were significantly ($P < 0.03$) lower than the ratios displayed by ND1 and ND5 genes. In both young and aged groups, the COX I expression is about twice COX III expression ($P < 0.01$).

The ND1/GAPDH ratio was significantly reduced ($P < 0.05$) in platelets from young subjects with respect to platelets from aged ones. A similar pattern was apparent for the ND5/GAPDH ratio ($P < 0.005$).

In order to exclude the possibility of artefacts in the PCR analysis, we have performed a parallel study on two pools from young and aged individuals (see Section 2) by real-time PCR and Northern blotting. The results of the real-time PCR are somewhat different with respect to the previous experiment on single individuals shown in Fig. 1 since they show a low expression level of the COX I and COX III genes in the pools of the young. Although we cannot compare these single values with those, statistically significant, of the previous set of experiments, it is worth noting that the real-time PCR data exactly overlap the Northern blot results for the same pools (Fig. 2).

4. Discussion

In this study we quantified the mRNA levels of selected mitochondrial genes in order to verify the effects of ageing on the mitochondrial transcription process. The interest of

this work stems from the use of platelets as pathogenic indicators and markers of ageing and diseases correlated to ageing [17], since they retain megakaryocyte-derived mRNAs and protein synthesis activity [21]. The mitochondrial transcript levels were investigated by real-time RT-PCR TaqMan, due to the small amounts of RNA that could be recovered from platelets from a normal blood withdrawal. Nevertheless, two pooled fractions of platelets from different individuals showed overlapping results by the alternative technique of Northern blotting.

Mitochondrial transcript levels of COX I and COX III subunits obtained by real-time RT-PCR analysis and normalized to GAPDH show that COX I expression is twice that of COX III in both young and old individuals. This result is surprising since mitochondrial mRNA transcription is polycistronic and the holoenzymes analyzed (Complex I and Complex IV) contain equivalent amounts of each subunit. It is therefore likely that some specific post-transcriptional regulation occurs or a different stability of the transcripts leads to this different steady-state level. These data are consistent with those published by Grossman et al. [22] showing that the expression of different COX genes is not regulated by the same set of factors. By contrast, our results are not consistent with data obtained from rat tissue. In fact COX I and COX III transcript levels have an inhomogeneous pattern in different rat tissues although COX I and COX III protein levels do not appear to be different [13].

Comparison of the transcript copy number in relation to ageing shows a significant increase of transcripts for Complex I ND5 and ND1 subunits during ageing. On the contrary, there is no significant change of transcript copy number for COX subunits. These results are different from those found by other investigators in rat tissues. The RNA species (12S rRNA and COX I mRNA)/DNA molecule ratio was significantly reduced in senescent brain and heart but was unchanged in liver [12], whereas in the study of Barazzoni [13] no significant changes or low reductions of COX I and COX III transcripts were found in different tissues from aged rats. However, Complex I subunit transcription was not investigated.

Complex I activity in platelets during ageing was studied in our laboratory: a significantly different sensitivity to the specific inhibitor rotenone was detected as an indicator of altered energy conservation [15]. In addition we found a bioenergetic mitochondrial deficiency in platelets from aged individuals using a functional test based on the Pasteur effect [18]. Since Complex I activity is rate limiting over the whole respiratory chain [23], it is possible that a higher expression of Complex I transcripts from platelets of aged individuals could compensate for the Complex I alteration. In general we can postulate that an upregulation in transcription of mitochondrial genes seems to compensate the OXPHOS disorders in human platelets during ageing.

Acknowledgements: The study was supported by grants from PRIN 'Bioenergetics: biophysical, biochemical and physiopathological aspects', MIUR, Rome, Italy.

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