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A comprehensive approach to determining BER capacities and their change with aging in *Drosophila melanogaster* mitochondria by oligonucleotide microarray

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ABSTRACT

DNA repair mechanisms are key components for the maintenance of the essential mitochondrial genome. Among them, base excision repair (BER) processes, dedicated in part to oxidative DNA damage, are individually well known in mitochondria. However, no large view of these systems in differential physiological conditions is available yet. Combining the use of pure mitochondrial fractions and a multiplexed oligonucleotide cleavage assay on a microarray, we demonstrated that a large range of glycosylase activities were present in Drosophila mitochondria. Most of them were quantitatively different from their nuclear counterpart. Moreover, these activities were modified during aging.

1. Introduction

The mitochondrial matrix is a rather harmful environment where strong oxidants are generated. These entities, termed reactive oxygen species (ROS), are produced continuously, mainly at the level of complexes I and III in the respiratory chain, during normal function, and are exacerbated during physical exercise or pathological conditions [1,2]. They are extremely detrimental to various cell structures such as proteins, lipids, and nuclear or mitochondrial DNA (mtDNA). mtDNA is directly bound to the inner membrane, and is less well protected than nuclear DNA [3]. ROS induce DNA base lesions through oxidation, deamination mechanisms and DNA strand breaks. Even though mtDNA molecules are numerous in every mitochondrion (average 2–10 copies), DNA lesions can accumulate and lead to heteroplasmy, a status where more than one type of mitochondrial genome is present in an organism. The vertebrate mtDNA is usually an extremely compact genome made up of 37 genes for essential respiratory complexes subunits, tRNA, and rRNA synthesis. Hence any DNA lesion can lead, with a high probability, to dramatic changes in mitochondrial protein expression and/or functions. Several pathologies have indeed been associated with mtDNA point mutations or deletions [4,5].

Mitochondrial DNA repair mechanisms have long been ignored or considered of low efficiency [6]. Today, mtDNA repair activities are fully acknowledged. They are efficient on single or double strand breaks in mitochondria [7–9], but mechanisms and actors remain mainly unknown. By contrast, base excision repair (BER) which is the main system handling the DNA damage resulting from oxidative insults activities, is well known and characterized in mitochondria [10,11]. Enzymatic proteins involved in this process are all nucleus-encoded. However, they are mostly identical to ac-

Abbreviations: AP site, abasic site; APE1, apurinic/apyrimidic endonuclease 1; BER, base excision repair; Cy3, Cyanine 3; Hx, hypoxanthine; NEIL1, Nei-like 1; 8oxoG, 8-oxo-7,8-dihydroguanine; NTH1, endonuclease three homolog 1; ODN, oligonucleotide; OGG1, 8-oxoguanine DNA glycosylase; PDH, pyruvate dehydrogenase; ROS, reactive oxygen species; Tg, thymine glycol; THF, tetrahydrofuran; U, uracil; UNG, uracil DNA glycosylase

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tive molecules found in the nucleus, or are isoforms from either alternative splicing or multiple transcription starting sites [12,13].

The different steps of BER are well described in [10] and [11]. The initial steps are base damage recognition and removal, through DNA glycosylases followed by abasic site recognition and hydrolysis by an endonuclease. But the entire specificity of the process is provided by the action of the different glycosylases.

Various mtDNA damages have been observed during pathological or physiological conditions. Among all of them, aging has been well studied leading to the hypothesis that mtDNA alteration could be a result of several processes: increase of damages causes, changes in replication activities or changes in mtDNA repair capacities [14]. Indeed, it has been shown that mtDNA damage increase and mitochondrial biochemical capacities decrease, during aging [14] putting the light on a potential involvement of BER activities in these phenomena.

Most studies on mtDNA repair mechanisms during aging have focused on either the whole process without discriminating specific glycosylases [15] or on single glycosylase activities [16,17]. Such analyses were performed by oligonucleotide (ODN) cleavage assays. In this process, ^{32}P-labeled oligonucleotides displaying various base alterations were used.

A more comprehensive assay allowing investigation of BER has been developed, ensuring simultaneous measurement of several excision activities [18–22]. This new approach was made possible by the use of microarrays functionalized by fluorescent lesion-bearing oligonucleotides. Only nuclear extracts have been tested so far using this multiplexed assay.

In this study, we show that these microarrays are very convenient for the quantification of BER activities in fruitfly mitochondria. This approach was used to analyze changes in mitochondria BER capacities during aging.

2. Materials and methods

2.1. Fruitflies

Fruitflies of *Drosophila melanogaster* strain w[1118] from the Bloomington Drosophila stock center, were reared in tubes (75 fruitflies per tube) at 19–20 °C on standard axenic medium [24]. Two populations of male fruitflies were used in this study: young (4–5 days after pupal hatching) and old (8 weeks after pupal hatching).

2.2. Subcellular fractionation

Reagents were from Sigma Aldrich unless otherwise stated. The entire procedure was performed at 4 °C in isolation buffer (IB): 10 mM HEPES, pH 7.6, 0.22 M sucrose, 0.12 M mannitol, 2 mM EGTA, 5 mM dithiothreitol, protease inhibitors (cOmplete Mini EDTA-free, Roche Diagnostic). Mitochondria and purified mitoplats were prepared according to protocols previously described in [24–26] and pure nuclear fractions were obtained as in [27]. The entire process was carried out as described in Fig. 1.

Protein concentrations were determined using the Bradford method [28] (Biorad).

2.3. Western blot analyses

Mitochondrial or nuclear fractions (15 μg proteins) were run on 10% or 15% (w/v) SDS–PAGE, blotted on nitrocellulose membrane (GeHealthcare) subsequently probed with primary antibodies raised against lamin (DSHB), histone H3, Rieske protein (gift from Dr. C. Godinot, Lyon) and pyruvate dehydrogenase (PDH, Mitosciences). Antibodies dilutions were respectively 1:750, 1:2000, 1/13 500 and 1:2000. Incubations and detections were carried out as previously described [23].

2.4. Enzyme assays

Cytochrome *c* oxidase and citrate synthase assays were performed on mitochondrial or nuclear fractions according to a previously described protocol [29]. All the enzymatic measurements were carried out using a heat-controlled spectrophotometer (Shimadzu) at 28 °C.

2.5. DNA repair assays

The excision reactions were conducted using the modified oligonucleotide arrays already described elsewhere [20,21]. The principle of these oligonucleotide arrays and oligonucleotides characteristics were detailed in a previous study [20]. Briefly, each well of a 24-well slide was functionalized by a series of lesion-containing oligonucleotide duplexes (lesion ODNs) and a control duplex (control ODN, with no lesion). The duplexes were tethered in duplicate to the slide surface by one end, and labeled with a Cyanine 3 (Cy3) at the other end.

Eight lesion substrates were present: ethenoadenine (EthA) paired with T, hypoxanthine (Hx) paired with T, 8oxoguanine (8oxoG) paired with C, A paired with 8oxoG, thymine glycol (Tg) paired with A, tetrahydrofuran (THF), as abasic site (AP site) analog, paired with A, uracil (U) paired with G and U paired with A.

Excision reactions were conducted with 10 μg of protein per well at room temperature (23 °C) for 1 h in 80 μL of excision buffer (10 mM HEPES/KOH pH 7.8, 80 mM KCl, 1 mM EGTA, 0.1 mM $ZnCl_2$, 1 mM DTT, 0.5 mg/ml BSA). The slides were subsequently rinsed for 3 × 5 min with 80 μL of washing buffer (PBS containing 0.2 M NaCl and 0.1% (v/v) Tween 20). On each slide, two wells were incubated with the excision buffer only (control wells) to serve as a reference, arbitrarily set at a fluorescence level of 100% for the calculation of the excision rates. The spot fluorescence was quantified using a GenePix 4200A scanner (Axon Instrument, Molecular Devices, Sunnyvale, CA, USA). Total spot fluorescence intensity was determined using the GenePix Pro5.1 software (Axon Instrument). Each extract was tested twice. The results between replicates (four spots) were normalized using the NormalizeIt software as described in [20]. A first operation calculated the excision rate of each of the lesion ODNs in the extract-containing wells compared with the lesion ODNs of the control wells. A correction was then applied that took into account the possible degradation of the control ODN (no lesion). For each slide, normalized fluorescence level of control wells (repair buffer alone) was taken as reference and fluorescence level of each lesion-ODN of control well was set up to 100. Final results for each lesion ODN, expressed as percentage of cleavage, were calculated using the formula: 100 × (1−percentage of fluorescence of lesion ODN/percentage of fluorescence of control ODN).

2.6. Statistical analyses

When histograms are presented, data are expressed as mean ± standard deviation (S.D.). Five to eight different extractions were performed and tested by enzymatic assays (each in duplicate) and on oligonucleotides microarrays (each in duplicate). Statistical significance was assessed using the Student's *t* test. For all statistical tests, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purified mitochondrial and nuclear fractions

As a first step prior to any repair measurement in mitochondria, we sought to obtain purified functional mitochondrial fractions with no contaminants from cytosol or nuclear compartments, in