The effect of aging and an oxidative stress on peroxide levels and the mitochondrial membrane potential in isolated rat hepatocytes

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Abstract We have investigated the effect of ageing and of adriamycin treatment on the bioenergetics of isolated rat hepatocytes. Ageing per se, whilst being associated with a striking increase of hydrogen peroxide in the cells, induces only minor changes on the mitochondrial membrane potential. The adriamycin treatment induces a decrease of the mitochondrial membrane potential in situ and a consistent increase of the superoxide anion cellular content independently of the donor age. The hydrogen peroxide is significantly increased in both aged and adult rat hepatocytes, however, due to the high basal level in the aged cells, it is higher in aged rat cells not subjected to oxidative stress than that elicited by 50 μM adriamycin in young rat hepatocytes. The results suggest that a hydrogen peroxide increase in hepatocytes of aged rats is unable to induce major modifications of mitochondrial bioenergetics. This contrasts with the damaging effect of adriamycin, suggesting that the effects of the drug may be due to the concomitant high level of both superoxide and hydrogen peroxide.

Key words: Hepatocyte; Oxidative stress; Adriamycin; Mitochondrion; Membrane potential

1. Introduction

A current hypothesis postulates that deleterious reactions initiated by partially reduced oxygen species are an important factor for the ageing process of multicellular organisms [1]. The initial molecular species of the univalent pathway of oxygen reduction is the superoxide anion radical which is converted into H_2O_2 either by spontaneous disproportionation or enzymatically by superoxide dismutase activity. H_2O_2 itself is a major source of cell damage by production of the extremely reactive hydroxyl (•OH) radical [2]. Therefore, the disposal of H_2O_2 by peroxidase- and catalase-catalyzed reactions is critical to prevent the accumulation of aggressive •OH radicals [3].

The main site of $O_2•^-$ and H_2O_2 production in eukaryotic cells is the electron transport chain in mitochondria [4,5], although there are uncertainties on the actual site and component responsible for one electron transfer to oxygen.

The role of oxygen radical-mediated injury in the cell ageing process might be critical. The free radical theories of ageing [1] attribute a critical role to reactive oxygen species (ROS) in the accumulation of random errors in macromolecules, responsible for the decline in cell performance. A special role has been ascribed to ROS-mediated somatic mutations of mitochondrial DNA (mtDNA) [6,7] in the energetic failure characterizing senescence. The mitochondrial theory of ageing [7,8] predicts that post-mitotic cells are maximally prone to accumulate mtDNA lesions and in fact, severe mtDNA deletions [9,10] and concomitant respiratory chain activity decreases [11–14] were described in heart, brain and muscle mitochondria of aged animals.

Liver cells, due to their mitotic potential, would be expected to be less prone to accumulate mtDNA mutations [15] since they are continuously renewed, defective cells would be competitively removed in favor of clones of energy-sufficient cells [16].

On the other hand, an additional reason why post-mitotic cells are considered to be preferentially affected by mtDNA mutations is their highly oxidative character [15], which renders them intrinsically more exposed to the continuous generation of reactive oxygen species. To this purpose, at difference with other mitotic cells, hepatocytes are very rich of mitochondria and have a high respiratory activity. Furthermore, their mitotic character is largely in a resting state except during active proliferation under regeneration conditions [17]. Previous studies do not appear to have solved this potential dilemma, since the literature reports conflicting data on the bioenergetics of liver in ageing [14,18–20].

For this reason, we have decided to undertake a study on isolated hepatocytes from livers of adult and old rats, aimed to look for ROS production and bioenergetic properties of their mitochondria. Besides basal conditions, we have considered it of interest to expose the cells to an oxidative stress in order to compare their defence potential and proneness to oxidative damage. The effect of adriamycin on perfused rat liver or on isolated rat hepatocytes was documented in previous studies [21,22] and appears to represent a suitable system to compare the sensitivity of young and aged animals to stress. Adriamycin is a potent drug in cancer therapy whose reduction to semiquinone by mitochondrial complex I [23] or by the microsomal P_{450} complex [24] results in a release of the superoxide anion and hydrogen peroxide.

For this reason, we have studied the effect of the adriamycin treatment on isolated hepatocytes of adult (6 months) and aged (24 months) rats, employing flow cytometry for probing the superoxide and hydrogen peroxide production and the mitochondrial membrane potential ($\Delta\Psi$mit) in situ.

2. Materials and methods

Two groups of male albino rats of the Wistar strain, aged 6 and 24 months, respectively, purchased from Stefano Morini (Italia), were

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kept for 1 week under constant environmental conditions and fed a normal laboratory diet. Each group was composed of eight animals.

Hepatocytes were isolated by a modification [25] of the two-step collagenase liver perfusion technique of Seglen [26]. The cell viability was determined by trypan blue exclusion or propidium iodide staining (5 μg/ml) for 5 min. Hepatocytes (1×10^6 cell/ml) were suspended in Krebs-Henseleit medium (pH 7.4) supplemented with 5 mM glucose under an atmosphere of 95% O_2/5% CO_2 in a shaking bath at 37°C and treated with different concentrations of adriamycin (from Sigma) for 2 h.

Flow cytometry analyses were performed with an EPICS C Cell Sorter (Coulter). Fluorochromes were excited at 488 nm with an argon laser at 200 mW. A forward angle light scatter (FALS) and right angle light scatter (90LS) were used to select live or dead hepatocytes [27]. The fluorescence was collected through a 488 nm blocking filter, a 560 nm long pass dichroic mirror and a 520 nm short pass filter (RH-123, DCHF-DA) or a 570 nm long pass filter (PI). Correlated histograms and listmode data were collected for linearly (FALS, 90LS) or logarithmically (three decades, 256 channels) amplified signals (RH-123, DCHF-DA, HE). The fluorescence of adriamycin [28] was used to determine the incorporation of the drug. The ΔΨmit was estimated by incubating hepatocytes (3×10^5 cell/ml) with 50 ng/ml rhodamine-123 (RH-123, Lambda Fluoreszenz-technologie, Graz, Austria) for 10 min at 37°C [29]. The cellular content of the superoxide anion was estimated by incubation of hepatocytes (3×10^5 cells/ml) with 10 μg/ml dihydroethidium (HE, Molecular Probes, Junction City, OR, USA) for 10 min at 37°C [30]. The cellular content of hydrogen peroxide was assayed by incubation of hepatocytes (5×10^5 cells/ml) at 37°C with 2 μM dichlorofluorescin diacetate (DCHF-DA, Molecular Probes) for 15 min [30]. The dichlorofluorescein (DCF) fluorescence units were converted to concentration units using a calibration curve, according to Bass et al. [31], slightly modified. Hepatocytes (5×10^5 cell/ml) were incubated with increasing concentrations of DCHF-DA (0.1–5 μM) for 10 min, to allow the uptake and deacylation of the probe and, after a treatment with 300 μM H_2O_2 for 30 min, the fluorescence intensity of the resulting DCF was measured by flow cytometry. Then, the sample was centrifuged at 4000 rpm for 5 min and the pellet was suspended in 2 ml of Krebs-Hanseleit medium, pH 7.4, and frozen at −80°C to break the cell plasma membrane. The supernatant obtained by centrifugation at 15000 rpm for 15 min was used to measure the DCF fluorescence intensity at 520 nm after excitation at 488 nm, using a JASCO 470 spectrofluorometer. The amount of DCF corresponding to the fluorescence intensity was extrapolated from a second calibration curve obtained by monitoring the fluorescence of the spectrophotometrically determined (503 nm) DCF concentration.

All data are presented as means ± S.D., the significance of differences was evaluated by the unpaired *t*-test and accepted when $P \leq 0.05$.

3. Results

The incorporation of adriamycin into rat hepatocytes is demonstrated following its fluorescence emission at 595 nm that increases linearly up to 100 μM adriamycin (Fig. 1). We have demonstrated that the intracellular adriamycin concentration becomes stable within 15 min. At this time, practically 100% of the cells exhibit a single peak of fluorescence, moreover, the adriamycin fluorescence does not interfere with that of RH-123 and DCF, whilst in the case of ethidium, the fluorescence of adriamycin was quantitatively subtracted.

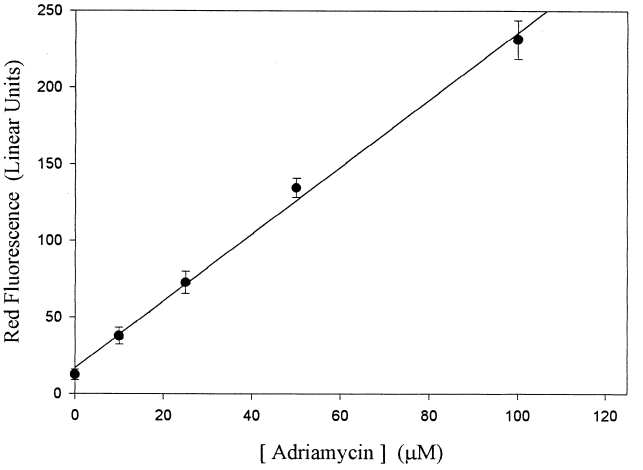

Fig. 1. The dose-dependent increase of adriamycin fluorescence at 595 nm (arbitrary units) in rat hepatocytes in a cytometric assay (see text)

The cellular viability is not significantly compromized in the presence of 50 μM adriamycin, whereas at concentrations equal or higher than 100 μM, the percentage of dead hepatocytes is higher than 25–30%.

The effects of adriamycin on hepatocytes from adult and aged rats are summarized in Table 1. The treatment with adriamycin induces a linear dose-dependent release of both superoxide anions, measured as the red fluorescence emitted after the oxidation of dihydro-ethidium, and peroxides, measured as the green fluorescence emitted after oxidation of 2′,7′dichlorofluorescein. The cellular content of superoxide anion is almost the same in adult (6 months) and aged (24 months) rat hepatocytes. Moreover, the increase of its content, induced by different concentrations of adriamycin, is not age-dependent and is 5–6-fold that of the controls in the presence of 100 μM drug.

On the other hand, the basal cellular amount of hydrogen peroxide is higher in aged (14-fold) than in adult rat hepatocytes: a typical cytometric diagram is shown in Fig. 2. The 50 μM adriamycin treatment (again shown in Table 1) induces a 6-fold and 4-fold increase of hydrogen peroxide in adult and aged rats, respectively. Even if the percentage increase is higher in adult than in aged rats, the effective concentration is much higher (10-fold) in the old ones.

In order to evaluate the effect of adriamycin treatment on

Table 1
The effect of adriamycin on the superoxide release, hydrogen peroxide production and mitochondrial membrane potential in hepatocytes from adult and old rats

Adriamycin μM	Adult			Aged		
	Superoxide release	Peroxide production	ΔΨmit	Superoxide release	Peroxide production	ΔΨmit
0	4.32 ± 2.20	3.65 ± 3.20	73.12 ± 29.79	4.77 ± 4.32	92.00 ± 42.76*	86.00 ± 47.37
10	8.18 ± 4.31	7.62 ± 2.24	48.30 ± 18.04	5.24 ± 2.31	157.41 ± 35.01	43.26 ± 25.16
25	9.38 ± 4.64	18.00 ± 7.57	32.38 ± 9.38	9.40 ± 2.86	222.50 ± 17.76	39.62 ± 18.90
50	17.15 ± 5.67	30.20 ± 9.88	23.86 ± 7.90	24.51 ± 9.27	427.21 ± 162.7	30.52 ± 12.76
100	28.71 ± 6.72	35.07 ± 10.1	18.47 ± 8.51	25.67 ± 10.2	351.0 ± 141.01	14.69 ± 5.97

See the text for explanations. Hydrogen peroxide is given in pmol/10^6 cells, all other parameters are in fluorescence arbitrary units.
*Significantly different from the peroxide production in adult hepatocytes.

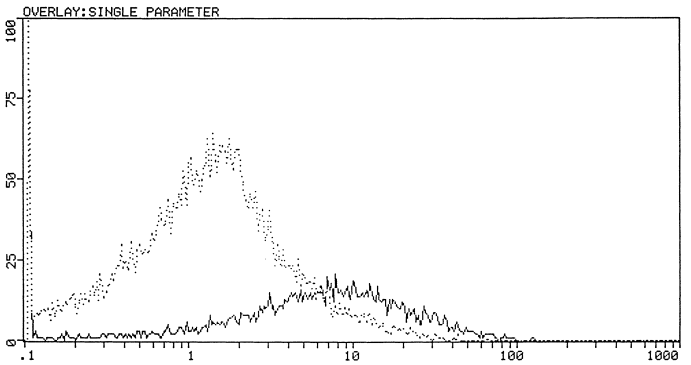

Fig. 2. A flow cytometric histogram (counts versus log fluorescence intensity) of the intracellular hydrogen peroxide content, measured as the 2′,7′dichloro-fluorescein fluorescence intensity ($\lambda_{em} \leq 520$nm) in hepatocytes from adult (dashed line) and old rats (continuous line).

the mitochondrial functionality, we have used RH-123, a fluorescent cationic dye which distributes electrophoretically into the mitochondrial matrix in response to $\Delta\Psi$ [29]. RH-123 is specifically incorporated in mitochondria. In fact, agents known to depolarize or de-energize mitochondria, such as uncouplers and respiratory chain inhibitors, decrease the RH-123 fluorescence of mitochondria in cultured cells, whereas agents which collapse the ΔpH and hyperpolarize $\Delta\Psi$, such as nigericin, increase the fluorescence. We have chosen our experimental conditions on the basis of previous data indicating that the cellular uptake of 50 ng/ml RH-123 reaches the steady state after 10 min of incubation [29]. In hepatocytes from both adult and aged rats, the $\Delta\Psi$mit is not significantly different. Adriamycin treatment of rat hepatocytes, independently on age, induces a significant dose-dependent decrease of $\Delta\Psi$mit (Table 1).

4. Discussion

The free radical [1] and mitochondrial [7,8] theories of ageing emphasize the importance of an unbalance in the rate of generation of ROS and of their removal. An increase of both superoxide anion and hydrogen peroxide with age was described in different species of flies [32] and in isolated rat hepatocytes [33], whereas other authors failed to observe an increased ROS production in ageing [34]. In this study, we have observed a striking increase of hydrogen peroxide but not of the superoxide anion in the isolated hepatocytes of aged rats. The reason may be either in an unbalance of the systems producing and consuming hydrogen peroxide or in the establishment of a steady state between the increase of the systems producing superoxide and of those disproportionating superoxide to peroxide. Irrespective of the reasons for this finding, the important conclusion is in the unbalance of the superoxide radical towards peroxide: such an unbalance is considered to be of potential danger, since it is peroxide that gives rise to the extremely aggressive hydroxyl radical [35]. Indeed, an increased peroxide to superoxide ratio has been considered an important aspect in degenerative diseases [36].

Adriamycin stress enhances both the superoxide and peroxide production. The extent of this enhancement is in the order of 2–6-fold for superoxide and the effect is identical in the cells from young and old animals. On the other hand, peroxide is increased to a moderately higher extent in the adult than in aged animals. Nevertheless, the basal content being a 14-fold higher in the aged rats, the peroxide levels reached in presence of adriamycin are dramatically elevated in the latter. This means that hepatocytes of old rats contain dangerous levels of hydrogen peroxide and are potentially subjected to undergo striking bursts of this damaging species and hence of its product, the hydroxyl radical.

In this study, we have found contrasting consequences of the rise of reactive species of oxygen at the mitochondrial level. To this purpose, we have to clearly distinguish between the effect of ageing per se and that of the induced oxidative stress.

In spite of the dramatic increase of hydrogen peroxide in the cells from aged rats, $\Delta\Psi$mit, investigated in situ by RH-123 and flow cytometry, is similarly high in cells from adult and old animals.

On the other hand, the induced oxidative stress after adriamycin addition induces a dramatic fall of the $\Delta\Psi$mit observed in the intact cell by flow cytometry. This impressing decrease is independent on the donor age. This observation is in good agreement with a cytoplasmic source of adriamycin-produced ROS. It is well established that the mitochondrial permeability transition, which occurs by opening of a large inner membrane pore and depolarizes the organelle [37], is induced by a variety of factors including oxygen radicals [38]. The outer mouth of the pore can be easily reached by hydrogen peroxide, produced at an extramitochondrial site, through the highly porous outer mitochondrial membrane. It is therefore suggested that mitochondrial depolarization in our system occurs by induction of the permeability transition. Alternatively, the permeability transition might be induced directly by binding of the drug to the outer mouth of the pore.

As a concluding remark, the findings in our study suggest that mitochondria in rat hepatocytes are not significantly affected by ageing. Even if cells from aged individuals have a dramatically higher content of hydrogen peroxide than cells from younger rats, this fails to induce important effects at the mitochondrial level. Acute production of this species, induced by adriamycin treatment, depolarizes mitochondria though not altering inner membrane activities to dramatic extents. It is puzzling that the basal peroxide levels in the old animals are higher than the highest levels induced by adriamycin in the young animals, yet they are not able to depolarize mitochondria. It is unlikely that the effect is due to a different compartmentation of the basal ROS production and of the adriamycin-induced production, since hydrogen peroxide is a diffusible species. An explanation may be in the different production of the most damaging hydroxyl radicals through the

Fenton reaction [2], which could be enhanced through the increased superoxide production by adriamycin treatment, but not by ageing. Finally, we cannot exclude a direct action of adriamycin on the permeability transition pore.

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