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Changes in adult rat liver mitochondrial populations at different energy states analyzed by flow cytometry

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Abstract

The present work studies the changes in green fluorescence intensity after Rh-123 staining of the low (LFP) and the high fluorescence populations (HFP) in isolated mitochondria from rat liver. The results show that the HFP represents a mitochondrial compartment less sensitive to changes in energy states. In addition, it is concluded that the use of Rh-123 to monitor changes in mitochondrial membrane potential should be undertaken with caution because, under certain circumstances, there is no correlation between the Rh-123 intensity of fluorescence due to its uptake by mitochondria and previously reported changes in the mitochondrial membrane potential.

Keywords: Rhodamine-123; Mitochondrion; Flow cytometry; Membrane potential; (Rat liver)

1. Introduction

In the past few years flow cytometry has become a potent and essential tool in the study of the physiology of the whole cell and its organelles [1–5]. This highly sensitive technique combines the advantages of both microscopy and biochemical studies in a rapid multiparametric analysis of large numbers of either individual cells or their organelles. In this sense, both intracellular and isolated mitochondria stained with membrane-permeable cationic lipophilic dyes that are known to selectively stain these organelles, such as rhodamine-123 (Rh-123), have recently been studied by flow cytometry [6–11]. Among other reasons, interest has focused on this dye because its uptake by mitochondria seems to be associated with its membrane potential [10–14]. Thus, it has been suggested that Rh-123 studies may offer a rapid and useful tool for the measurement of mitochondrial membrane potential [9–11,15,16].

We have recently reported the existence of two different mitochondrial populations detected by flow cytometry when isolated rat liver mitochondria are stained with Rh-123: a low fluorescence population (LFP) and a high fluorescence population (HFP) [17]. The proportion between these populations is not stable; the LFP increases progressively from newborn rats to adult age during 'in vivo' development [18] and in 'in vitro' cultured hepatocytes [19]. In addition, the ratio between both mitochondrial populations is affected by the energy state of mitochondria from adult rat liver [18]. Moreover, the in vivo interconversion between these two mitochondrial forms during the early postnatal period depends on both cytosolic and mitochondrial protein synthesis [20]. These results suggest that both populations are functionally different and that the HFP may represent a more immature compartment of mitochondria.

In the present work we studied the Rh-123 uptake of these two rat liver mitochondria populations at different energy states in order to analyze whether or not energization affects Rh-123-uptake in both mitochondrial populations as measured as the fluorescence intensity due to Rh-123 on the basis of a single mitochondrion. Our results indicate that the HFP is less sensitive to changes in the mitochondrial energy status as assessed by their fluorescence intensity due to Rh-123-uptake, suggesting that they may represent a functionally less differentiated mitochondrial compartment.

Abbreviations: FCCP; carbonyl cyanide *p*-(triluoromethoxy)phenylhydrazone.

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2. Materials and methods

2.1. Mitochondrial isolation and respiratory measurements

The whole mitochondrial fraction (WMF) was isolated from the liver of Wistar rats (250 g body wt.). Liver was homogenized with 5 ml of isolation medium (250 mM sucrose, 1 mM EDTA and 10 mM Hepes (pH 7.4)) per 1 g of tissue. 2 ml of this homogenate was layered onto a discontinuous Percoll gradient based on the method reported by Reinhart et al. [21] with some modifications [17]. The density of WMF measured by coloured density-marker beads (Pharmacia LKB, Uppsala, Sweden) was 1.082 g/ml. In agreement with Pertoft and Laurent [22], isolation with Percoll did not interfere with mitochondrial viability. Respiratory measurements were carried as has been previously described [17].

2.2. Mitochondrial rhodamine-123 staining

In order to correlate the energy status with Rh-123-uptake, 1.0–1.4 mg of mitochondrial protein was placed in a respirometer (Oxymeter, Gilson Medical Electronics, France) cell (2 ml) to measure oxygen consumption and

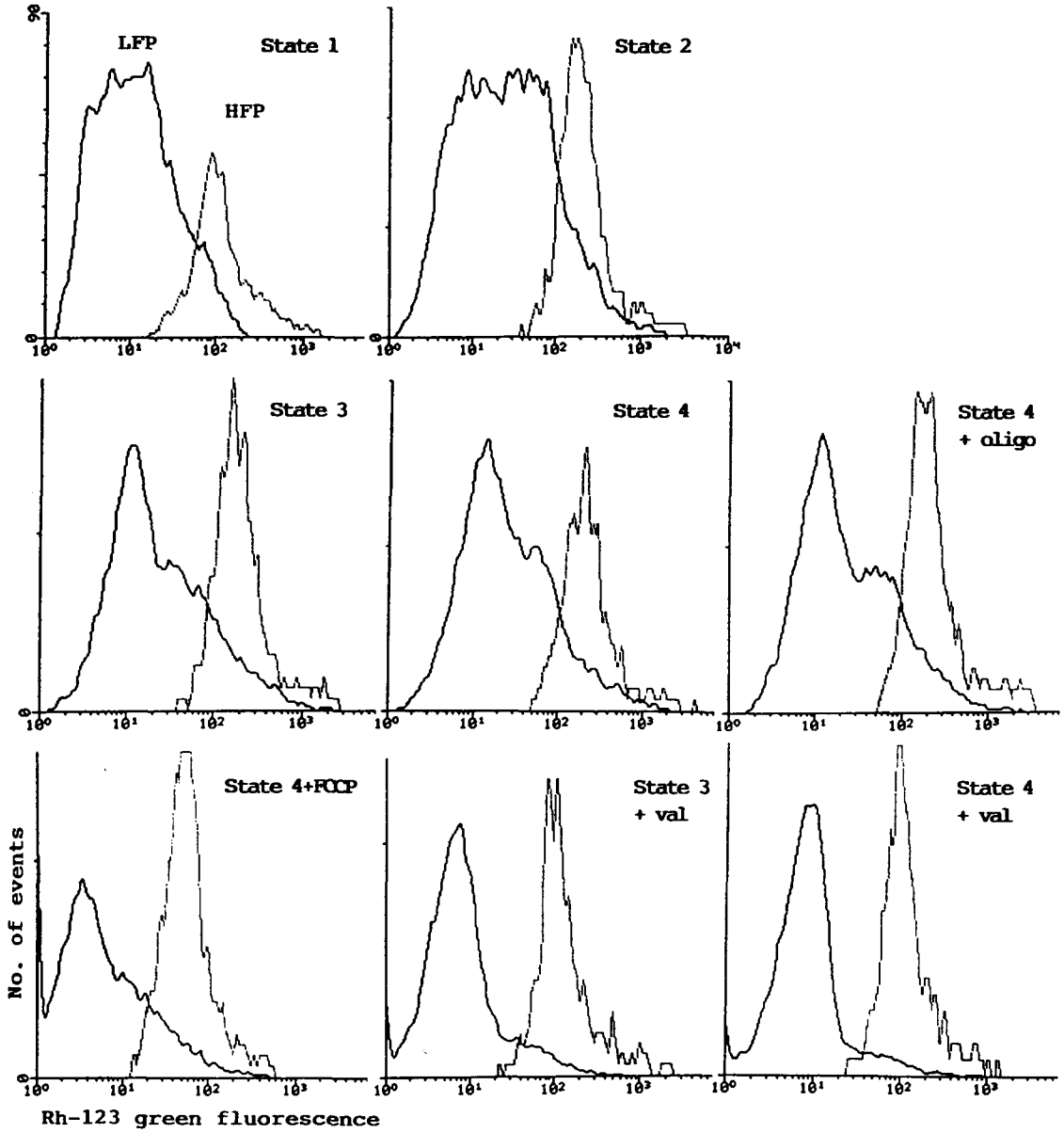

Fig. 1. Rh-123 green fluorescence (log scale) histograms of low (LFP) and high fluorescence (HFP) populations of whole mitochondrial fraction. Bold line: LFP; plain line: HFP. State 1: mitochondria in respiratory medium; State 2: State 1 plus substrate (succinate 10 mM); State 3: State 2 plus 400 nmol ADP; State 4: after all ADP is converted to ATP; State 4 + oligomycin (80 ng); State 4 + FCCP (2 μM); State 4 and State 3 + Val (0.5 μM).

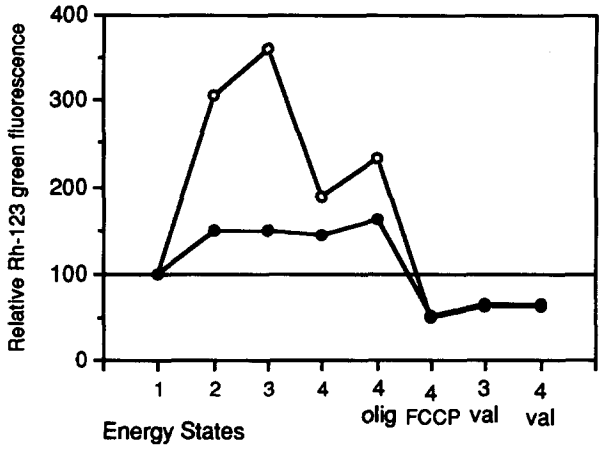

Fig. 2. Relative Rh-123 green fluorescence of low (LFP) and high fluorescence (HFP) populations of the whole mitochondrial fraction at different energy states. Mean fluorescence channels of both LFP and HFP. State 1 values were taken as 100%. LFP: open circles; HFP: closed circles. For energy states description see legend of Fig. 1.

Rh-123-uptake in the same sample. For this purpose, after measuring oxygen consumption, an aliquot of 1 ml (0.5–0.7 mg protein) was discharged into an Eppendorf tube containing 20 μl of Rh-123 1.25 mM, the final concentration being 25 μM. ADP (3 mM), oligomycin (80 ng/ml) and FCCP (2 μM) were added to state 2, state 4 and state 4 plus oligomycin mitochondria, respectively. Valinomycin (0.5 μM) was added to both state 3 and state 4 mitochondria. Mitochondrial Rh-123 staining was performed as previously reported [17]. In all cases, rhodamine-123 staining was performed independently from oxygen consumption measurements.

2.3. Flow cytometry mitochondrial analysis

All measurements of mitochondrial fluorescence and 90° angle light scatter (SSC) were made for at least 10000 events/test with a FACStar flow cytometer using the Consort 30 software program (Becton/Dickinson, San José, CA, USA). The data on mitochondrial fluorescence and light scatter were obtained using a 5 W argon ion laser tuned at 488 nm and 250 mW, and Rh-123 green fluorescence was measured through a 530 ± 15 nm band pass filter placed in front of the green photomultiplier tube using a four-decade log scale. The mean intensity fluorescence channel for each histogram and for each population were determined using the Consort 30 program. Graphics were plotted by means of the Lysys 1.0 software (Becton/Dickinson). All experiments were performed within a 30 min time period, using the same instrumental set up and conditions in order to obtain comparable results for the different measurements.

2.4. Statistical analysis

Mean channels for each histogram and for each population were determined using the Consort 30 software program (Becton/Dickinson). Statistical analyses of mean fluorescence intensity of LFP and HFP populations were performed using a normal distribution [23].

3. Results

As has been reported before [17], two distinct mitochondrial populations were obtained upon analysing the rat liver whole mitochondrial fraction at flow cytometry: a low fluorescence population (LFP) and a high fluorescence population (HFP). These populations were present in all the energy states analyzed (Fig. 1).

Table 1 shows the mean intensity of fluorescence and the HFP/LFP ratio at different energy states. Fig. 2 shows the relative fluorescences of both HFP and LFP mitochondrial populations. The mean fluorescence per mitochondrion increased in both the LFP and the HFP when substrate (state 2) was added to mitochondria in respiratory medium. However, this increase was relatively higher in the LFP since the ratio between the intensities of fluorescence of the LFP and the HFP decreased to almost one half of its initial value (Table 1 and Fig. 2). When ADP was added to state 2 mitochondria (state 3), a slight increase in the mean fluorescence intensity of the LFP was observed, while no major changes were detected within the HFP. In a similar way, at state 4 a significant decrease in

Table 1
Rhodamine-123 mean green fluorescence channels of low (LFP) and high fluorescence populations (HFP), and HFP/LFP ratios of whole mitochondrial fraction

Energy states								
State	1	2	3	4	4 + Oligo	4 + FCCP	4 + Val	3 + Val
LFP	20 ± 0.04	62 ± 0.8 *	73 ± 0.6 *	38 ± 0.3 *	47 ± 0.4 *	10 ± 0.02 *	13 ± 0.02 *	13 ± 0.03 *
HFP	180 ± 1.3	268 ± 2.1 *	268 ± 2.3	257 ± 2.1 *	291 ± 2.3 *	94 ± 0.3 *	116 ± 0.4 *	116 ± 0.04 *
HFP/LFP	9	4.3	3.7	6.7	6.2	9.4	8.9	8.9

Results are expressed as mean channel ± S.E.M. (n = 10000). State 1: mitochondria in respiratory medium; State 2: plus substrate (succinate 10 mM); State 3: plus 400 nmol ADP; State 4: after all ADP has been converted to ATP; State 4 + Oligo: plus 80 ng oligomycin; State 4 + FCCP: plus FCCP 2 μM; State 4 and State 3 + Val; plus valinomycin 0.5 μM. State 2 is compared against state 1; state 3 against state 2; state 4 against state 3. States 4 + Oligo, 4 + FCCP, 4 + Val, 3 + Val are compared against state 4 and state 3, respectively. * $P < 0.001$.

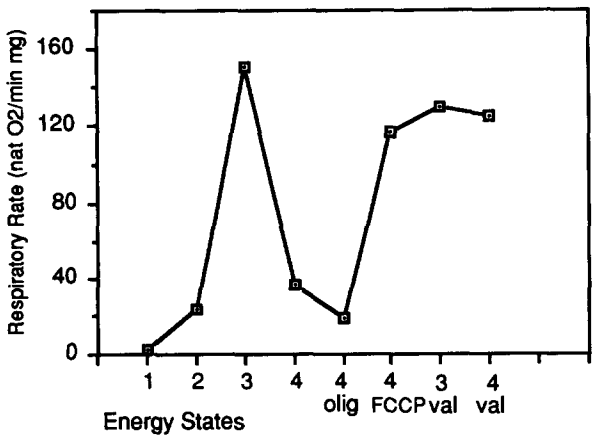

Fig. 3. Representative experiment of respiratory rate (nat O_2/min mg protein) of whole mitochondrial fraction with succinate (10 mM) as substrate. For energy states description, see legend of Fig. 1.

the mean fluorescence of the LFP was found while a minor decrease in the HFP was detected (Table 1 and Fig. 2). Thus, the mean ratio between the fluorescence intensity of both populations approached its initial values (Table 1). Upon adding oligomycin to the state 4 mitochondria, a slight increase in the mean intensity of fluorescence of both the LFP and HFP was observed, no major changes being detected in the ratio between both populations (Table 1 and Fig. 2).

Finally, the presence of an uncoupling agent such as FCCP induced a dramatic decrease in mitochondrial fluorescence, this specially affecting the LFP and thus leading to a fairly high HFP/LFP ratio (Table 1 and Fig. 2). When valinomycin was added to either state 4 or state 3 mitochondria, a significant decrease in the mean fluorescence intensity of the two mitochondrial populations was observed (Table 1 and Fig. 2), the ratio between the HFP and the LFP mean fluorescence intensities being as high as its initial values (Table 1).

Fig. 3 shows the respiratory rate at the different energy states. Oxygen consumption increased slightly from the respiratory medium (State 1) to state 2 (with succinate), reaching its maximum when ADP was added (state 3). When all the ADP had been converted to ATP, a dramatic decrease in oxygen consumption was detected, this being slightly decreased in the presence of oligomycin. Deenergization agents such as FCCP or valinomycin induced a strong increase in oxygen consumption as previously reported.

4. Discussion

It has recently been shown that isolated adult rat liver mitochondria split into two different mitochondrial populations which are identifiable at flow cytometry on the basis of their Rhodamine-123 uptake, i.e., a low (LFP) and a high fluorescence population (HFP) [17].

The percentages between the LFP and the HFP vary significantly from the newborn to the adult rat, a progressive increase in the LFP being observed during in vivo development [18] and in in vitro culture [19]. This results could reflect the occurrence of different types of cell in the liver. However, it has been shown that both rhodamine-123-stained mitochondrial populations are present in primary culture hepatocytes [19], which are free of other liver cells since they are grown in an arginine-free medium that prevents proliferation of cells lacking ornithine carbamyltransferase (EC 2.1.3.3). This would support the notion that, instead of being related to the existence of different liver cell types, the occurrence of LFP and HFP mitochondrial populations would reflect the existence of distinct functional types of mitochondrion [17]. Accordingly, LFP would represent a more mature form of mitochondria that becomes more frequent as the animal develops. In addition, it has been demonstrated that the ratio between both mitochondrial populations changes according to their energy status [18] and depends on both cytosolic and mitochondrial protein synthesis [20]. These data suggest that the proportions of the two mitochondrial populations would be a dynamic parameter that would somehow allow the assessment of the functionality of mitochondria. However, to date, no efforts have been made to directly analyze whether or not these two mitochondrial populations show different degrees of functionality.

Like other energy-transducing organelles, mitochondria are able to generate a high proton electrochemical gradient according to the Mitchell chemiosmotic hypothesis [24]. This gradient includes both a membrane potential and a ΔpH, the former predominating [25]. The membrane potential of mitochondria has been shown to change according to the energy status and the magnitude of its changes reflects the functionality of mitochondria [10,26]. Accordingly, the membrane potential increases strongly when a substrate is added to mitochondria in respiratory medium. The addition of ADP to state 2 mitochondria (state 3) induces a decrease in their membrane potential that is associated with an increase in oxygen consumption [10,26], both the membrane potential and oxygen consumption return to previous levels when all the ADP has been converted to ATP (state 4). The addition of oligomycin on state 4 mitochondria slightly increases their membrane potential [26]. Finally, when either state 3 or state 4 mitochondria are placed in the presence of either uncoupler or ionophore agents, such as FCCP or valinomycin respectively, a dramatic decrease in mitochondrial membrane potential occurs that is associated with a strong increase in oxygen consumption [10,26].

Rhodamine-123 is a membrane-permeable lipophilic cationic dye that has been shown to accumulate in cells, cell organelles and liposomes exhibiting an internal negative membrane potential [16]. It has been shown that

Rh-123 can be used to monitor the membrane potential in cells [2,5,16,27], isolated mitochondria [10,28,29] and chloroplasts [30]. In agreement with this, we observed (Table 1) that the mean intensity of fluorescence due to Rh-123 uptake by isolated rat liver mitochondria increases from state 1 to state 2, this being detected in both the LFP and the HFP. By contrast, the presence of either FCCP or valinomycin, which depolarize the mitochondrial membrane by dissipating ΔpH or $\Delta\Psi$, respectively, strongly decreases the mean intensity of green fluorescence due to Rh-123 uptake by the LFP and HFP; the effect of valinomycin was identical in both state 4 and state 3. In addition, upon adding oligomycin to state 4 mitochondria a slight increase in the mean fluorescence intensity of both the LFP and HFP mitochondria was detected; this being in consistent with the reported increase in the mitochondrial membrane potential under these conditions [10,11]. These results largely support the notion that the changes in Rh-123 fluorescence reflect, at least to a certain extent, the variations in the mitochondria membrane potential. However, it should be mentioned that some discrepancies were observed in our study between the changes observed in Rh-123 fluorescence and the expected variations associated with the energy status. Accordingly, our results show that the decrease in mitochondrial membrane potential in state 3 [10,26], with respect to that of state 2 and 4 (which are functionally the same), is surprisingly associated with a slight increase in the mean intensity of fluorescence of the LFP, but with no changes in that of the HFP. These data would indicate that, although the changes in Rh-123 intensity of green fluorescence correlate to a certain extent with those of the mitochondrial membrane potential, under certain conditions discrepancies may be observed. Previous studies have shown that following mitochondrial energization, Rh-123 displays a red shift in both its absorbance [29,31] and fluorescence spectrum, together with a quenching of its fluorescence [29]. Accordingly, the addition of ADP to state 2 mitochondria induces a decrease in the fluorescence quenching with an increase in total fluorescence, although changes in both the absorption and emission spectra of Rh-123 are unimportant [29]. This would probably explain why the changes from state 2 to state 3 are consistently associated with a slight increase in the mean intensity of green fluorescence of Rh-123-stained LFP mitochondria even though after addition of ADP to state 2 there is a decrease in their membrane potential.

It was of interest to analyze whether or not identical changes in the mean fluorescence intensity of the LFP and HFP mitochondria would appear at different energy states. For this purpose, the ratio between the mean fluorescence intensity of the HFP and LFP was calculated. Our results show (Table 1) that, while during energization there is a decrease in this ratio, the presence of ionophores such as FCCP and valinomycin is associated with a high HFP/LFP mean florescence intensity ratio. These data suggest that, when compared to the HFP, the LFP is a much more sensitive mitochondrial population with respect to its energy states as assessed by the mean fluorescence intensity due to Rh-123 uptake. Accordingly, our results would support the notion that, from the functional point of view, the HFP mitochondria from adult rat liver are less differentiated as compared to the LFP mitochondria.

In summary, our results show that measurements of the mean intensity fluorescence of Rh-123 staining on isolated adult rat liver mitochondria to monitor their membrane potential should be taken with caution and that under certain conditions discrepancies may appear. In addition, it is shown that the HFP display a lower ability to react to changes in their energy status as compared to the LFP, supporting the idea that the HFP may represent a more immature mitochondrial compartment.

Acknowledgements

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