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Effect of Adriamycin treatment on the lifetime of pyrene butyric acid in single living cells

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Abstract

We investigated the fluorescence lifetime of pyrene butyric acid (PBA) using various O_2 concentrations in cells. Both in living and freshly fixed cells, PBA lifetime decreased with oxygen concentration. We recorded decay curves in single cells and measured PBA lifetime and NAD(P)H intensity values. Under nitrogen atmosphere, the probe lifetime differences (199 and 209 ns in living and freshly fixed cells, respectively) suggest a supplemental pathway for the deactivation of the probe when the cell functions are not stopped. We propose reactive oxygen species (ROS) to be the additional quenchers that cause this decrease. We further studied the effect of drugs generating ROS the anthracycline doxorubicin (adriamycin). For living cells, PBA lifetime decreased after adriamycin (ADR) treatment (200 and 1000 ng/ml). This supports our hypothesis that under nitrogen atmosphere and for freshly fixed cells, PBA lifetimes increase to an unchanging value due to absence of quenchers.

Keywords: *Oxygen, reactive oxygen species, lifetime, pyrene butyric acid, adriamycin, living cells*

Introduction

In the early seventies Vaughan and Weber [1] first described oxygen quenching of 1-pyrene butyric acid (PBA) fluorescence in solution. At room temperature without oxygen, the pyrene cycle has a lifetime of a few hundred nanoseconds. This property, due to the symmetry of the molecule, is exploited to detect oxygen concentration through the probe quenching. PBA was used to study the diffusion of oxygen in plasma membranes [2]. It is nowadays used for membrane fluidity detection [3] and oxygen sensing film [4]. Other pyrene derivatives allow measurement of oxygen concentrations in living cells by the detection of fluorescence intensity variations [5,6] or fluorescence lifetime changes [7]. Lifetime measurements are not dependent on the absolute intensity of the emitted light and, therefore, independent of the probe concentration. This property is advantageous when the probe is loaded into living cells. Our interest in the oxygen measurement in living cells lead us to develop new probes derived from pyrene [8] and to compare them with PBA [9]. Both studies indicate differences between living cells and model membranes. It was then put forward that a supplemental pathway for the deactivation of PBA in living cell, other than oxygen quenching, is needed when PBA lifetime was compared to the lifetime obtained in fixed cells [10]. We suggest that free radicals could affect the lifetime of PBA in living cells. Indeed, it was demonstrated earlier that free radicals can act as fluorescence quenchers of probes such as pyrene derivatives [11,12] in solution. In living cells, the mitochondrial electron transport chain accounts for most of the oxygen consumed in a cell. A small part of this oxygen is incompletely metabolized and diverted into superoxide anion. This superoxide anion subsequently generates species such

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as hydroxyl radicals and hydrogen peroxide which together are commonly called reactive oxygen species (ROS) [13,14].

Adriamycin (ADR) has been one of the most often used anthracyclines against a variety of solid and hematological tumors for the last three decades. Due to its quinone ring, ADR is known to generate ROS through an enzymatic pathway (flavine-reductases) [15–18] or a non-enzymatic pathway involving a ferric ion [18–20]. In the present work, we use this capacity of ADR to generate ROS in living cells.

The main objective of this work is to test the hypothesis that the presence of free radicals can affect the lifetime of PBA. We used the CCRF-CEM-WT cell line to study PBA lifetime and intrinsic NAD(P)H intensity under different conditions. A control population is build from about hundred living cells. Cell fixation allows stopping cell function together with ROS generation. We studied control populations of living and freshly fixed cells under air atmosphere and nitrogen atmosphere. Because anthracyclines were previously reported to generate free radicals, we used this ADR capacity to demonstrate the involvement of ROS in PBA lifetime decrease. We compared PBA lifetimes in control cells to PBA lifetimes in cells treated with two ADR concentrations.

Materials and methods

Chemicals

1-pyrenbutyric acid (PBA) was purchased from Acros organics (Geel, Belgium), dissolved in ethanol at the concentration of 50 μM and stored at 4°C. RPMI 1640 was purchased from Cambrex (Verviers, Belgium), and Hanks' balanced salts solution (HBSS) was purchased from Sigma Chemical Co (St Louis, MO, USA). A stock solution of Adriamycin (ADR) (Sigma, St Quentin Fallavier, France) was prepared into appropriate concentrations (30 and 300 μg/ml) in Phosphate-buffered saline solutions and stored at −20°C.

Cells and culture conditions

Human leukemic cell line (lymphoblasts) CCRF-CEM-WT provided by Dr W.T. Beck (College of Medicine, University of Illinois at Chicago, Chicago, IL) were grown as stationary suspension cultures in medium RPMI 1640 supplemented with 2 mM L-Glutamine, 10% heat-inactivated fetal calf serum and 1% of antibiotics (penicillin and streptomycin), at 37°C in a humidified chamber (5% CO_2). The cells were seeded every three or four days at 2×10^5 cells/ml to maintain exponential growth.

Stainning Procedure

ADR treatment. Cells were treated, by adding appropriate drug volume into 5 ml cell culture medium containing 2×10^5 cells/ml, to reach final drug concentrations of 200 and 1000 ng/ml. Drug treatment lasted for 4 days and measurements were carried out each day. For each treatment condition, the measurements were repeated on triplicate samples.

PBA loading. Cells suspension containing 2×10^5 cells in HBSS was incubated with PBA (1% ethanol) at the concentration of 0.6 μM, at 37°C with 5% CO_2 during 20 min. The cells suspension was centrifuged and rinsed twice. The cells were re-suspended in HBSS, and laid on Sykes-Moore chamber for observations. For fixation experiments, after rinsing, the cells were fixed with 10% paraformaldehyde in 1% calcium chloride aqueous (Baker) solution. Cell fixation time did not exceed 30 min to avoid probe reorganization [10].

Cell growth inhibition. Toxicity experiments were performed on CCRF-CEM cells grown in a complete culture medium containing PBA 0.1–100 μM (1% ethanol) at 37°C with 5% CO_2. Two replicates of each concentration were used in each assay. Cell counts were performed in duplicate.

Fluorescence lifetime apparatus

The apparatus used has been described elsewhere [8]. A nitrogen laser (LSI VSL 337 ND) delivers monochromatic pulses at a wavelength of 337 nm. The beam is concentrated by mean of a reflecting objective (74 ×) (Ealing Electro-Optics, Watford, England) on a microscopic sample. Emitted photons are collected and focused on a photomultiplier (RCA 1P28). The signal is digitized by a numerical oscilloscope (Tektronix TDS 350). A 404 nm band-pass filter (half bandwidth 40 nm) is placed on the emission path to select the pyrene emission. A neutral density filter (70%) is added on the excitation path, allowing reduction of laser beam intensity. With this filter, several repeated measurements on a same cell do not induce detectable photobleaching.

Cell atmosphere control

Fluorescence lifetime measurements were achieved under air atmosphere and nitrogen atmosphere. Absence of oxygen in the cell atmosphere was obtained using a flowmeter (Aalborg, NY, USA) supplying nitrogen gas. Nitrogen flow was regulated at 80 ml/mn. The gas, after humidification by bubbling through water, arrived under a lid designed to cover the Sykes-Moore chamber. Nitrogen gas was flushing above cells suspension, and a stable decay was usually obtained after 10 min. Fluorescence lifetime measurements were achieved between 21 and 24°C. A pressure of 1 atm was maintained during the experiments.

Analysis of the Results

The fluorescence response $S(t)$ is considered as the convolution product of $G(t)$, the apparatus response for excitation pulse, and $s(t)$, the impulse response of the fluorescence: $S(t) = G(t) \times s(t)$. The apparatus response $G(t)$ can be simulated with a Gaussian curve convoluted with a time constant. The parameters of the Gaussian curve and the time constant are obtained after analysis of the 1,4-di-[2-(5-phenyloxazolyl)]-benzene solution response. In most cases, $s(t)$ can be defined as the sum of exponentials with time constant τ_i and amplitude A_i. The time constants and amplitude values are determined either by the modulating functions method [21] or by iterative reconvolution using a non-linear least-squares fit method [22]. Correct fits are chosen by examination of the residual curve, the difference between the experimental and theoretical curves, and for the lowest value of the estimator Q_2. This estimator is the average of the residual square values.

Statistical analysis. A amount of 6–8 single cells were investigated for each cell population sample. Each experiment was repeated 3–6 times. The number of cells is accessible in both tables. The mean and standard deviation were calculated from all the decay values. The error on lifetime evaluation stems from (i) the systematic error in data acquisition and data treatment, and (ii) by statistical fluctuations of the signal. Since we are using comparative measurements, with the same acquisition and treatment system, the systematic error will be roughly the same for all measurements. Therefore, the uncertainty of our lifetime evaluation mainly stems from the statistical noise of the signal (standard deviation). In order to reduce the standard deviation we averaged 100 to 200 fluorescence decays. The standard deviation for repeated measurements on a given solution was found to be <1 ns. Measurements performed with cell populations, heterogeneous samples, showed standard deviations of around 5 ns. This value roughly characterizes the cell population heterogeneity.

Results

Decay curves

The decay curves are recorded on a single living cell (Figure 1). The curve of fluorescence of the cell without probe (Figure 1A), selected by a 404 nm filter, corresponds to the intrinsic fluorescence of the cell attributed to reduced form of nicotinamide adenine dinucleotide phosphate (NAD(P)H) (fluorescence range 400–650 nm). The decay curve is roughly fitted with a single exponential decay and the associated lifetime is about 6–9 ns. A more accurate

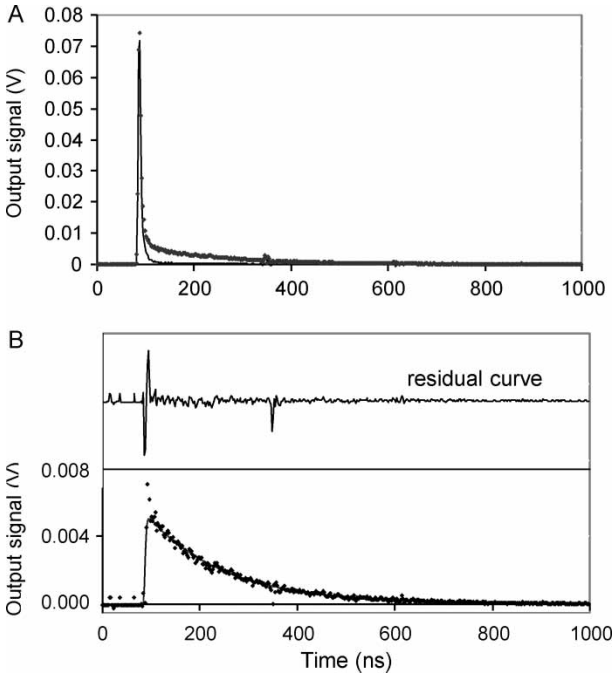

Figure 1. Fluorescence decay profiles under air atmosphere. (A) Fluorescence decay observed in CCRF-CEM-WT cell loaded with PBA (0.6 μM) (◆), intrinsic fluorescence decay in a single living cell (solid line). (B) Fluorescence decay curve of the probe obtained by subtraction of the two previous curves (◆). The solid line through the decay curve shows the calculated single exponential curve. On the top of the figure is shown the associated weighted residual curve.

analysis shows bi-exponential decay with first lifetime shortly below 1 ns for free NAD(P)H and ~8 ns for bound NAD(P)H as expected [23]. The signal intensity is proportional to total NAD(P)H concentration. A comparable NAD(P)H fluorescence intensity is obtained when the cell is loaded with 0.6 μM probe (Figure 1A). The decay curve is best fitted with a double exponential decay. The first decay corresponds to the intrinsic fluorescence of the cell. The longer decay corresponds to the probe fluorescence. The long lifetime (158 ns) is characteristic for pyrene derivatives. However, in order to obtain this lifetime value, we use a curve (Figure 1B) resulting from the subtraction of the decay obtained for a single cell without PBA from the decay obtained for a cell loaded with PBA. The resulting single exponential decay corresponds to the decay curve of PBA alone. The lifetime τ is then calculated by fitting the curve (Figure 1B). All the decay constants available in tables are determined by this method.

Measurements on control cells

Recorded with a 404–nm filter, the decay curves of cell loaded with PBA give two main informations: PBA decay constant and NAD(P)H fluorescence intensity. NAD(P)H intensity can be related to the

Table I. PBA lifetimes (τ) and NAD(P)H fluorescence intensities ($I_{NAD(P)H}$) obtained for living cells and freshly fixed cells populations. Measurements are carried out under air atmosphere and nitrogen atmosphere (N_2), after 3 days cells growth.

	Atmosphere conditions							
	Living Cells				Freshly Fixed Cells			
	PBA loaded		Without PBA		PBA loaded		Without PBA	
	Air	N_2	Air	N_2	Air	N_2	Air	N_2
τ(ns)	158 (3)	199 (4)	—	—	169 (4)	209 (3)	—	—
$I_{NAD(P)H}$ (a.u.)	74 (17)	77 (20)	73 (15)	79 (12)	39 (12)	34 (11)	30 (13)	30 (14)
Cells number	94	68	16	12	65	66	10	10

Means and standard deviations (in parenthesis).

energetic state of cells. A large number of cells is studied to obtain statistical values. The mean and standard deviation of the population are given in Table I. The distribution of these two data sets, obtained for living cells loaded with PBA, is represented in Figure 2. In Figure 2A the histograms of PBA lifetime is shown in single living cells under air atmosphere (mean: 158 ns) and under nitrogen atmosphere (mean: 199 ns). As expected without oxygen, the lifetime of the pyrene derivative increases, according to the Stern-Volmer equation as previously shown [10]. In Figure 2B, the intensity of NAD(P)H fluorescence is shown for living cell loaded with PBA under air atmosphere and under nitrogen atmosphere. The mean values are similar (74 and 77 a.u., respectively). Neither PBA loading nor nitrogen flow changes the NAD(P)H fluorescence intensity under our experimental condition (Table I).

Measurements on freshly fixed cells

Table I summarizes the values obtained for living cells and cells fixed by baker solution. After short time fixation, the cell structure organization is similar to the organization in living cells. The cell activity is stopped without cell necrosis, which is confirmed by no-coloration with blue trypan. The similarity of the cell structure for living and freshly fixed cells is assured by less than 30-min fixation (freshly fixed cells) according to [10]. The mean value of the NAD(P)H fluorescence intensity is reduced to 30 a.u. for freshly fixed cells. No clear change appears when the cells are placed under nitrogen atmosphere or loaded with PBA. This decrease of NAD(P)H fluorescence intensity is consistent with the ceasing of the cell activity. Simultaneously, PBA decay curves are registered for freshly fixed cells. Under air atmosphere, PBA lifetime reaches 169 ns. It increases to 209 ns under nitrogen atmosphere. The lifetime is still sensitive to the quenching by oxygen. However these lifetimes are higher than those for living cells (158 and 199 ns). This can be explained, as described earlier [10], either by changes of the probe environment or by disappearance of additional quenchers in fixed cells. In the latter case, the second quencher is supposed to be generated during the cell activity. It disappears when the cells are fixed allowing the lifetime reaching a higher lifetime value.

NAD(P)H fluorescence intensities in ADR treated cells

The NAD(P)H fluorescence intensity are measured daily during 4 days on cells treated with ADR (200 and 1000 ng/ml). In Figure 3, the data are compared with the non-treated cells intensity (control cells). The experiments are performed with living cells and with freshly fixed cells. These data are obtained under air atmosphere. They are roughly similar to the data

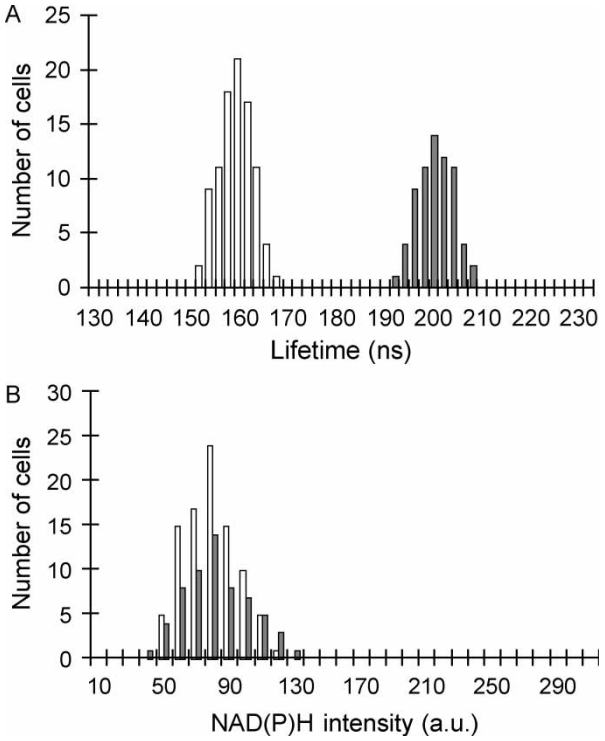

Figure 2. Histograms of (A) PBA fluorescence lifetime, (B) intrinsic fluorescence of the cells at 404 nm (i.e. NAD(P)H intensity). *Y*-axis corresponds to the number of cells. Data are measured under air atmosphere (hollow histograms) and under nitrogen atmosphere (full histograms) for 3-days cell growth.

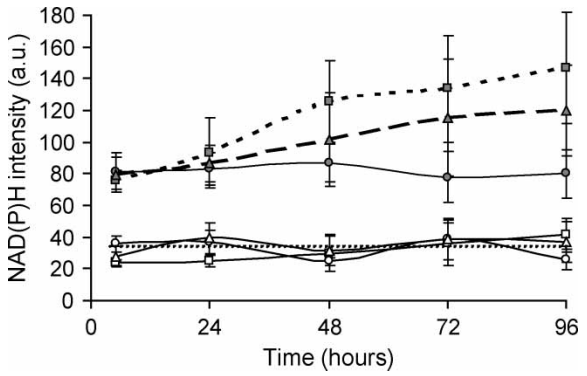

Figure 3. Variation of the NAD(P)H fluorescence intensity with treatment time for cells treated with 200 ng/ml (□) and 1000 ng/ml (Δ) ADR concentration and for control cells (○). The values were obtained under air atmosphere in living cells (full symbols) and in freshly fixed cells (hollow symbols).

obtained under nitrogen atmosphere. For the living control cells, NAD(P)H fluorescence intensity remains constant during the 4 days (80 a.u.). As mentioned above, the intensity decreased after fixation (30 a.u.) for control and treated cells. In contrast, there is an increase of NAD(P)H fluorescence intensity for living cells treated with 1000 ng/ml ADR. The increase is even higher for 200 ng/ml adriamycin. Significant differences are only seen after two days treatment (125 a.u. for 200 ng/ml and 101 a.u. for 1000 ng/ml). After 4 days, the intensities reach 147 and 120 a.u. respectively. Is this increase of NAD(P)H fluorescence intensity related to an increase of intracellular NAD(P)H concentration ? Or in other word is it related to an increase of the cells metabolic activity, or to an increase of the cell size without change of NAD(P)H concentration ? It has been shown [25] that the 200 ng/ml ADR dose induced polyploidy (80% of the cell population after 3 days) with very large increase of the mean cell size. In contrast, the 1000 ng/ml dose blocked the cell population growth, without changing the population mean cell size. In order to compare intracellular NAD(P)H concentrations we weighted the mean fluorescence intensity of a cell population by the corresponding mean cell surface. The results, $R_{NAD(P)H\text{-}S}$, are given in Table II. For the control cells this ratio is 0.119. This ratio increased considerably for 1000 ng/ml treatment from 0.166, after 3 days to 0.169 after 4 days. However, there was no significant increase for 200 ng/ml treatment (0.120 and 0.130 respectively). Thus, the intracellular NAD(P)H level increased only for the 1000 ng/ml treatment. Furthermore, this increase was detectable only between 24 and 48 h treatment (Figure 3).

PBA lifetimes in ADR treated cells

The PBA fluorescence decays are recorded on living or freshly fixed cells treated with 200 or 1000 ng/ml ADR. The measurements are performed under nitrogen atmosphere. Thus, decreasing intracellular oxygen and simplifying the deactivation processes. Figure 4 shows NAD(P)H fluorescence intensity versus PBA lifetime after 1 day (Figure 4A) and 3 days (Figure 4B) treatments. After fixation, for treated or control cells, the data are similar. PBA lifetimes reach 210 ns and NAD(P)H fluorescence intensities remain around 30 a.u. For living cells, at day 1 (Figure 4A), the data are similar for control cells and for 200 ng/ml treatment (199 ns and 80 a.u.). On the contrary for the 1000 ng/ml ADR concentration, PBA lifetimes decrease down to 189 ns and NAD(P)H fluorescence intensity does not significantly vary (100 a.u.). As expected, the results for control cells at day 3 are identical to those at day 1. PBA lifetimes for 1000 ng/ml treated cells at day 3 (Figure 4B), are similar to those recorded at day 1. In contrast, NAD(P)H fluorescence intensities have increased (120 a.u.). For 200 ng/ml ADR concentration, PBA decay constant decreases at day 3 and reaches a value (188 ns) identical to the 1000 ng/ml treatment value. The NAD(P)H fluorescence intensities clearly increase (155 a.u.). Altogether, the *Y*-axis high points dispersion has increased (standard error: 40) compared to non- treated cells (s.e. 20) and to 1000 ng/ml ADR (s.e. 27).

PBA lifetimes under nitrogen are illustrated in Figure 5. During the four-day experiments, the

Table II. Average values of NAD(P)H fluorescence intensities ($I_{NAD(P)H}$) and cell surface area (S) obtained for control cells and for cells treated with two adriamycin (ADR) concentrations. Standard deviations are represented in parenthesis. From these values, the ratio $I_{NAD(P)H}/S$ is calculated: $R_{NAD(P)H\text{-}S}$. Measurements are performed in CCRF-CEM-WT cells after 2, 3 and 4 days cell growths.

	Time of treatment (days)	Cells number	$I_{NAD(P)H}$	S*	$R_{NAD(P)H-S}$
Cell control	3	68	78 (17)	660(150)	0,119
	2	20	126 (26)	–	–
ADR	3	47	133 (34)	1100 (330)	0,120
200 ng/ml	4	14	147 (36)	1130 (450)	0,130
	2	16	101 (30)	–	–
ADR	3	22	115 (37)	690 (150)	0,166
1000 ng/ml	4	15	120 (28)	710 (180)	0,169

* From J. Savatier [24] under the same condition of experimentation.

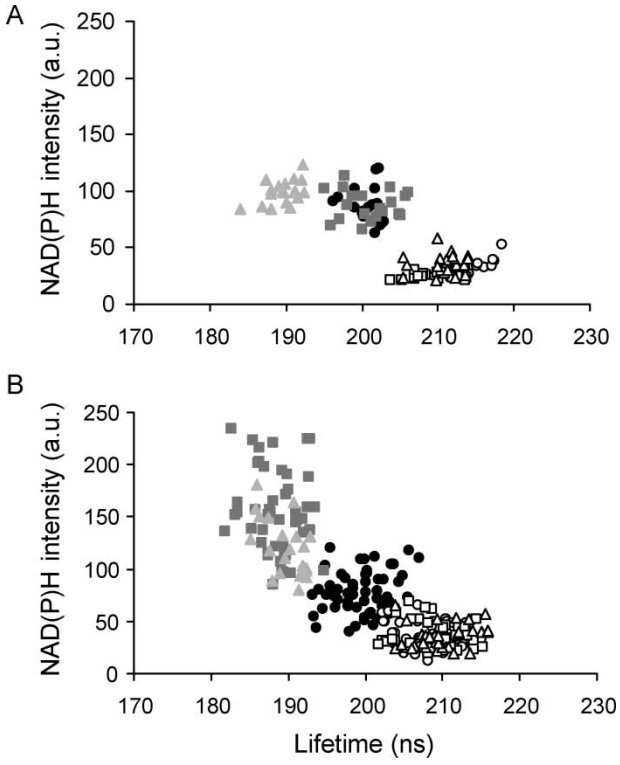

Figure 4. Representation of NAD(P)H fluorescence intensity as a function of PBA lifetime. Measurements are realized under nitrogen atmosphere in control cells (○) and in cells treated with 200 ng/ml (□) and 1000 ng/ml (Δ) ADR concentration after 1 day (A) or 3 days (B) treatment. Data are obtained from living cells (full symbols) or freshly fixed cells (hollow symbols).

lifetimes remained about quasi-constant for control cells (~200 ns). Likewise, PBA lifetime for 1000 ng/ml drug, remained constant throughout the time period of the experiment but with a smaller lifetime (~190 ns). Treated cells at 200 ng/ml have PBA lifetimes varying from those of the control cells for the first two days to those of the treated cells at 1000 ng/ml after 3 days. The ADR drug effect on PBA lifetimes is instantaneous for 1000 ng/ml treatment and delayed to the third day for 200 ng/ml treatment.

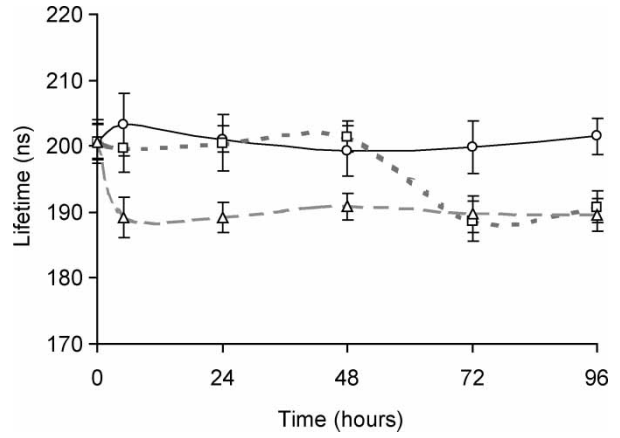

Figure 5. Variation of PBA lifetime with treatment time for cells treated with 200 ng/ml (□) and 1000 ng/ml (Δ) ADR concentration and for control cells (○). The values were measured under nitrogen atmosphere in living cells.

Discussion

PBA lifetime and NAD(P)H fluorescence intensity correlation

The fluorescence decay analysis is performed on a large number of cells. PBA lifetime depends upon quenchers concentration. NAD(P)H fluorescence intensity is related to NAD(P)H concentration. Mainly generated by the glycolitic pathway, NAD(P)H is used in mitochondria through the respiratory chain to generate the energy necessary to cell ATP production. Therefore, NAD(P)H fluorescence intensity allows us to follow NAD(P)H fluctuation and to gain information on the energetic cell status and activity. NAD(P)H fluorescence intensity is measured on PBA-free and PBA-loaded cells under both air atmosphere and nitrogen atmosphere. The intensity remains stable when loading PBA on living cells (Table I). This result is coherent with the fact that no cell growth inhibition is observed following PBA treatment. Under nitrogen atmosphere, NAD(P)H fluorescence intensity does not change for at least the 30 min of the experiments. Neither the PBA loading nor the oxygen removing from the top of the cells disturbed the cell activity. On the other hand, PBA lifetime changed with oxygen concentration. The dependence of PBA lifetime versus oxygen concentration was earlier described in details [10]. We conclude that the two parameters, PBA decay constant and NAD(P)H fluorescence intensity are not dependent upon each other. At low concentrations, PBA does not affect cell behavior.

PBA fluorescence quenching

Lifetime differences were determined under nitrogen atmosphere between freshly fixed cells (209 ns) and living cells (199 ns). Differences were first determined recently on 3T3 cells [10]. The lifetime increase in fixed cells can be explained by (i) change of the probe surrounding; (ii) disappearance of quenchers other than O_2. Since in freshly fixed cells the redistribution of the probe is unlikely, we believe that no change of the environment of the probe happens before two hours. Indeed, the fixation by baker is known to keep the membranes integrity. On the contrary, we have shown in a previous study [10] that for long fixation time (12 h) the probe localization changes. Furthermore, when the redistribution happens, the surrounding change has opposite effect on PBA lifetime (decrease up to 140 ns). Here we obtained indirect confirmation of a second quenching in living cells. The hypothesis stems from the noteworthy increase of

PBA lifetime under nitrogen atmosphere in freshly fixed cells. In fixed cells, the cell activity is stopped altogether with the generation of transient molecules (NAD(P)H-ATP-ROS). Under nitrogen no more quenchers are nearby and PBA lifetime is maximal. Even if the nature of the second quencher is not yet completely clarified, ROS are good candidates, as it is already known to quench PBA lifetime [11,12]. We investigated this hypothesis with chemotherapy antracycline drug.

PBA quenching related to ADR treatment

ADR drug treatment is known to increase ROS concentration [26]. We use two concentrations of this drug to test our hypothesis. Obviously we observe PBA lifetime decrease after both adriamycin treatments. The decrease confirms the increase of the non-radiative desactivation pathway. If ROS are PBA quenchers, ROS concentration increase will have such effect: PBA lifetime will decrease. For fixed cells, the cells activity and consequently ROS formation are stopped. Under nitrogen, the corresponding PBA lifetime is maximum and stable during the four-day experiment. The value is similar for control and treated fixed cells ($\sim$210 ns). Thus indicating that PBA environment remains comparable whenever fixation takes place. Moreover, for living cells, the PBA lifetime decreases do not appear simultaneously for both ADR concentrations. The decrease is instantaneous for 1000 ng/ml treatment. It appears after 3 days for 200 ng/ml treatment. ROS increase seems to be delayed for the lower drug concentration treatment. We then addressed the question, whether these decreases are due to an increase of the energetic cell potential and consequently to an increase of ROS production, or to direct ADR effects. For 1000 ng/ml ADR treatments, the PBA lifetime decrease occurs before the first 4 h, while the increase of the intracellular NAD(P)H level happens only after 24 h. For the 200 ng/ml dose, we did not observe a significant increase of intracellular NAD(P)H concentration and the decrease of PBA lifetime occurred after 3 days. Thus, the decrease of PBA lifetime observed for ADR treated cells cannot be related to a change in the cells energetic state. Consequently, the decrease is likely to be due to a direct effect of ADR on ROS generation through different pathways [15–20].

In conclusion, the experiments with living cells and cells fixed with a baker solution indicate that PBA lifetime increases when the cell activity is stopped. We attribute this lifetime increase to the disappearance of quenchers that had been generated by cell activity. We suggest that ROS diminution increases PBA lifetime. In the subsequent experiments the cells are treated with ADR. This drug is known to generate ROS. We observed a significant decrease of PBA lifetime after ADR treatment. This suggests that ROS production decreases PBA lifetime. In the near future, we intend to relate PBA lifetime with ROS concentration by studying drug that decrease ROS production in different points of the mitochondria oxidative chain and to determine which of the ROS are involved in the PBA lifetime reduction.

Acknowledgements

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