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Essential requirement of reduced glutathione (GSH) for the anti-oxidant effect of the flavonoid quercetin

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Abstract

We have analyzed the anti- or pro-oxidant effects of the flavonoid quercetin (QU) by evaluating, in U937 cell line, hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), reduced glutathione (GSH) content, mitochondrial membrane potential, DNA content, phosphatidylserine exposure on the outer face of the plasma membrane and cell viability. Polychromatic flow cytometry was used to evaluate in the same cells several functional parameters. For short periods of treatment QU exerted an anti-oxidant effect (decrease in H_2O_2 levels), whereas for long periods it showed a pro-oxidant activity (increase in O_2^-). In these conditions, GSH content was reduced, and this correlated with a lack of anti-oxidant activity of QU, which in turn could be correlated with proapoptotic activity of this molecule. Thus, QU can exert different effects (anti-/prooxidant) depending on exposure times and oxidative balance, and in particular on stores of GSH.

Keywords: *Quercetin, ROS, GSH, flow cytometry, apoptosis, mitochondrial membrane potential*

Abbreviations: *QU, Quercetin; H_2O_2, Hydrogen peroxide; O_2^-, Superoxide anion; GSH, Reduced glutathione; $\Delta\psi_m$, Mitochondrial membrane potential; PS, Phosphatidyilserine; ANX-V, Annexin-V; JC-1, 5,5′,6,6′tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; H_2DCF-DA, 2,7-Dicholorodihydrofluorescein diacetate; MBB, Monobromobimane; HE, Hydroethidine; PI, Propidium Iodide; FSC, Forward scatter; SSC, Side scatter*

Introduction

Reactive oxygen species (ROS) are continuously produced during cell metabolism. Under normal conditions they are scavenged and converted to non-reactive species by different intracellular, enzymatic and non enzymatic, anti-oxidant systems (catalase, peroxidase, superoxide dismutase, vitamins, reduced glutathione (GSH)) [1]. If there is an overproduction or an ineffective elimination of ROS, they can react with proteins, lipids and nucleic acids causing several cell damages and eventually cell death. In fact, ROS are involved in the etiology of degenerative diseases, coronary artery disease, stroke and cancer [2,3]. ROS also acts as signalling molecules important for signal transduction [4,5]. In particular, H_2O_2 can induce activation of NFκB, a transcription factor involved in several cellular processes [6].

Mitochondria are the most important intracellular sources of ROS. Indeed, nearly 4% of the oxygen used by the respiratory chain is incompletely reduced and can generate ROS [7]. This organelle is particularly vulnerable and therefore exposed to ROS injury. ROS accumulation causes lipid peroxidation and/or an

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increase in permeability known as mitochondrial membrane permeability transition which leads to apoptosis [8,9].

Flavonoids are a class of naturally occurring benzo-γ-pyrone derivatives which are present in fruits and vegetables [10]. In the last few years they have been widely analyzed because of their patho-preventive effects, including anti-allergenic, antiinflammatory, anti-proliferative and anti-viral actions [11,12]. These molecules show an apparent opposite double action: they seem to be at the same time pro-oxidant and anti-oxidant in different models. In fact, the mechanisms of these multiple effects have not yet been completely clarified [13–16].

Quercetin (3,3′,4′,5,7-pentahydroxyflavone, QU) is one of the most abundant dietary and frequently studied flavonoids [17]. The strong anti-oxidant effect of QU can be attributed to the high number of hydroxyl substituent groups and to its conjugated π orbitals by which QU is able to donate electrons or hydrogens [18]. Previous studies have shown that when QU reacts with ROS, it forms potentially harmful oxidation products. In cells, the oxidation products are inactivated by different molecules such as GSH, the major intracellular reducing agent, and ascorbate. In their presence, QU preferentially reacts with GSH to form two non reactive products: 6- glutathionylQuercetin (6-GSQ) and 8-glutathionylQuercetin (8-GSQ) [19].

Many flavonoids have cytostatic properties and may induce apoptosis. These effects are attributed to their modulation of several biological processes [20,21]. In particular, (some of) these molecules seem to exert a pro-apoptotic action that alters mitochondrial permeability [22]. Dissipation of the mitochondrial membrane potential can be an early event in the apoptotic process, followed by cytochrome c release and subsequent activation of caspase 3 [23]. QU can halt the growth of tumor cells by arresting them in different phases of the cell cycle [24,25]. QU blocks signal transduction pathways by inhibiting protein tyrosine kinase, PI and PIP-kinases (1- phosphatidylinositol 4-kinase and 1-phosphatidylinositol 4-phosphate 5-kinase) [26]. In addition to an antiproliferative action, QU causes apoptosis in several tumor cell lines. In fact, prolonged exposure to high doses of QU resulted in formation of the characteristic ladder of DNA fragments revealing activation of programmed cell death [27].

In the present study, we also measured the effects of QU on ROS content. To better clarify the anti/pro-oxidant role of QU, we analyzed U937 cells using a multiparametric flow cytometry approach that allowed us to measure at the same time H_2O_2, O_2^- and GSH content. We also evaluated cell viability and early or late apoptosis, in order to understand the relationship between the anti/pro-oxidant effect of QU and its ability to induce apoptosis.

Materials and methods

Cell line (U937) and culture conditions

Micoplasm-free U937 (monoblastic cell line) were grown at the density 5×10^5 cells/ml in complete culture medium (CM), i.e. RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Gibco BRL, Life Tecnologies, Scotland) at 37°C in humidified atmosphere (5% CO_2 in air). The cells were treated with QU (Sigma-Aldrich, St. Louis, MO, USA) 10, 50 and 100 μM for different periods of time: 2, 4, 6, 12, 16 and 24 h. After treatments, cells were collected, pelleted and stained with different fluorochromes and analyzed by multi-parametric flow cytometry, as described below.

Flow cytometry

Cytofluorimetric analyses were performed using a CyFlow ML (Partec GmbH, Münster, Germany), equipped with a solid—state laser (488 nm, 200 mW), a UV Mercury lamp HBO (100 long life, 100 W), a red diode laser (635 nm, 25 mW), a Nd:YAG laser (532 nm, 50 mW) and a CCD camera. A minimum of 10,000 cells per sample were acquired and analyzed by FloMax 3.0 or by WinMDI 2.8 softwares.

Analysis of DNA content. Hoechst 33342 (Molecular Probes, Eugene, OR) has the capacity to enter into living cells and to bind in a stoichiometric manner DNA sequences made of 3 AT base pairs [28,29]. Its use allows the recognition of apoptotic and viable cells. When Hoechst 33342 is excited by UV-lamp, it emits at 540 nm, and its signal is collected in the channel devoted to fluorescence 8 (FL-8).

Detection of phosphatidylserine (PS) on the outer face of plasma membrane. During the early phases of apoptosis PS is translocated from the inner to the outer leaflet of the plasma membrane, so that it is exposed to the external cellular environment [30]. PS exposure was detected by Annexin-V (ANX-V) labeled with Alexa Fluor 647 (Molecular Probes), which is excited by the red laser and emits at 665 nm (detected in FL-4).

Analysis of mitochondrial membrane potential ($\Delta\psi_m$). Cells were stained with the $\Delta\psi_m$-sensitive probe 5,5′,6,6′tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1, Molecular Probes), used at a final concentration of 10 μM. JC-1 is a lipophilic carbocyanine that exists in a monomeric form and is able to accumulate in mitochondria. In the presence of a high $\Delta\psi_m$, JC-1 can reversibly form aggregates that, after excitation at 488 nm, emit in the orange/red channel (in FL-2). The collapse in $\Delta\psi_m$ provokes a decrease in the number of JC-1 aggregates, and a

subsequent increase of monomers that emit in the green channel (FL-1). This phenomenon is revealed by either a decrease in FL-2 and an augmentation in FL-1 and, in a typical dot plot obtained by flow cytometric analysis, cells with depolarized mitochondria move from the upper left to the lower right quadrant of the panel [31,32].

Detection of reduced glutathione (GSH). Cells kept in phosphate buffered saline (PBS) were stained with monobromobimane (MBB, from Molecular Probes), which reacts nonenzymatically with GSH, binding the thiol-groups. When excited by UV lamp, MBB emits at 450 nm (detected in FL-8), with an intensity that is proportional to GSH content [29,33].

Detection of superoxide anions (O_2^-). Cells were stained with hydroethidine (HE, Molecular Probes) at the concentration of 1 μM. The probe reacts with O_2^- to give ethidium, which binds DNA and emits red fluorescence (detected in FL-2) when excited by a blue laser [34,35].

Detection of hydrogen peroxide (H_2O_2). Intracellular H_2O_2 content was measured by the probe 2,7-dicholorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes). H_2DCF-DA is lipid soluble and penetrates into the cells where it is transformed by non specific esterases into an intermediate H_2DCF. In the presence of peroxidase and H_2O_2, H_2DCF is converted to the fluorescent molecule 2,7- dichlorofluorescein (DCF) which, excited at 488 nm, emits at 520 nm (detected in FL-1) [36]. Cells were stained with H_2DCF-DA at the concentration of 2 μM [37].

Analysis of cell viability. Cells were stained with propidium iodide (PI, Sigma-Aldrich, St. Louis, MO) at the final concentration of 5 μg/ml in PBS, or of 0.25 μg/ml when used with Hoechst 33342. Necrotic and late apoptotic cells are characterized by alterations of plasma membrane permeability, and therefore PI penetrates into the cells, binds DNA and emits at 617 nm (in FL-3) when excited at 488 nm.

Multiparametric flow cytometry. The simultaneous use of the aforementioned fluorescent probes with dedicated protocols allowed us to analyze several parameters in the same intact cell. In particular, we performed the:

- *Analysis of DNA content, H_2O_2 production, early and late apoptosis and necrosis with Hoechst 33342, H_2DCF-DA, PI and ANX-V*. Cells were resuspended in RPMI 1640 and stained with 5 μM Hoechst 33342 for 30 min at 37°C, then centrifuged at 200*g* for 5 min at room temperature to remove unbound Hoechst 33342, resuspended in PBS and stained with H_2DCF-DA for 30 min at 37°C. After this incubation, cells were centrifuged at 200*g* for 5 min at 4°C, resuspended in 195 μl of Annexin binding buffer (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4) added with 5 μl of Alexa Fluor647-conjugated ANX-V. Cells were incubated for 10 min at room temperature, washed with 800 μl PBS and resuspended in 1.5 ml of Annexin binding buffer. PI was added 2 min before acquisition at low concentration (0.25 μg/ml) in order to avoid resonance energy transfer to Hoechst 33342 [38].
- *Analysis of GSH content, H_2O_2 and O_2^- production with MBB, H_2DCF-DA and HE*. During the last 45 min of incubation at 37°C with QU, HE was added to cell cultures, then cells were collected and centrifuged at 200*g* for 5 min at 4°C. Cells were resuspended in 1 ml of PBS and stained with H_2DCF-DA for 20 min at 37°C. MBB was then added and cells incubated for 10 min at 37°C. Before flow cytometric analysis, cells were centrifuged at 200*g* for 5 min at 4°C and resuspended in 1.5 ml of PBS.
- *Analysis of GSH content, $\Delta\psi_m$ and cell viability with MBB, JC-1 and PI*. Cells were stained with JC-1 for 10 min at 37°C, centrifuged at 200*g* for 5 min at 4°C, and then 1 ml of PBS with MBB (50 μM) was added. After 10 min at 37°C, cells were centrifuged as previously reported and resuspended in 1.5 ml of PBS. PI was added 2 min before sample acquisition at the concentration of 5 μg/ml.

Statistical analysis

Data are presented in the histogram as the median of the net fluorescence value ± S.E.M. The net fluorescence value was obtained: (i) linearising the fluorescence values from the logarithmic scale and (ii) subtracting the linearised median value of the blank (i.e. the sample containing cells treated in the same manner, but without any staining) from the median fluorescence value of the stained sample [39]. This allows to eliminate any influence related to the eventual autofluorescence. Statistical analysis was performed on Graph Pad Prism 3.03 using two-way ANOVA test. Values of $p < 0.05$ were considered as statistically significant.

Results

QU decreases intracellular H_2O_2 content

H_2O_2 content was measured by H_2DCF-DA in U937 cells incubated for 2, 4 and 6 h with QU 10, 50 and 100 μM (Figure 1). As shown in Figure 1a, the

fluorescence emitted by H_2DCF-DA, which is directly proportional to the amount of H_2O_2 present in the cell, was progressively reduced in the presence of increasing doses of QU, even after 2 h of incubation. The effect was particularly evident at the doses of 50 and 100 μM, and was well maintained after 4 (Figure 1b) and 6 h (Figure 1c) of incubation. These data indicate that QU exerts an anti-oxidant effect, particularly at high doses, towards basal H_2O_2 content.

In order to have direct evidence of this effect, H_2O_2 was added to cell cultures: U937 were pre-incubated for 2 h with QU (100 μM), washed and exposed to 50 μM H_2O_2 for 15 min. As shown in Figure 1d, cells treated with H_2O_2 alone (green line) showed an increased fluorescence when compared to untreated cells (black line). As expected, U937 cells pre-incubated with QU (red line) showed a diminished H_2O_2 content. When QU-treated cells were exposed to H_2O_2 (blue line), their fluorescence was much lower than that of cells treated with H_2O_2 alone, and was similar to that of untreated cultures. The same effect was also found for the two lower doses of QU (data not shown), and demonstrated the ability of QU to reduce H_2O_2 content.

These experiments were repeated a minimum of three times. Either the actions of QU on basal levels of H_2O_2 or to H_2O_2 added to the cultures were statistically significant, as shown in Figure 2a and b, respectively.

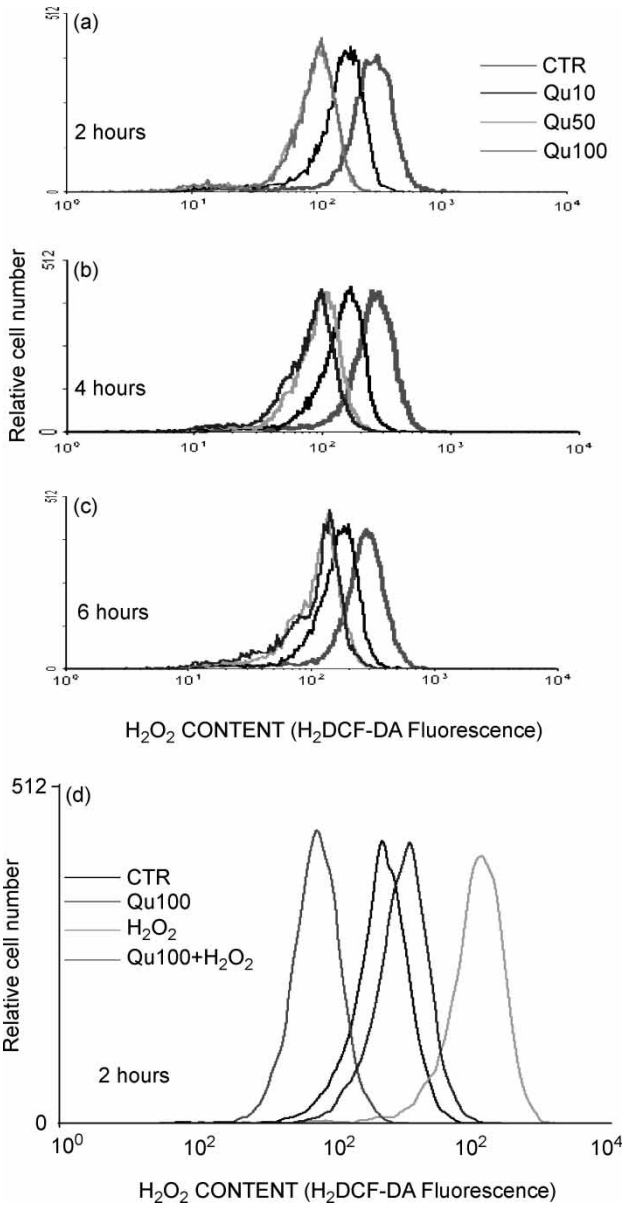

Figure 1. Flow cytometric analysis of U937 after H_2DCF-DA staining. U937 were incubated for 2(a), 4(b) and 6 h (c), respectively) in absence (CTR) or presence of QU 10, 50, 100 μM. (d) U937 were pre-incubated in absence (CTR) or in presence of QU 100 μM for 2 h, and then in the presence of H_2O_2 during the last 15 min.

QU-induced reduction of H_2O_2 content is not due to its toxicity

To be sure that the reduction of H_2DCF-DA fluorescence measured in cells incubated with QU was due to a real anti-oxidant capacity of QU and not to the presence of metabolically inactive cells, i.e. not viable cells, we used a multiparametric cytofluorimetric analysis that allowed us to discriminate

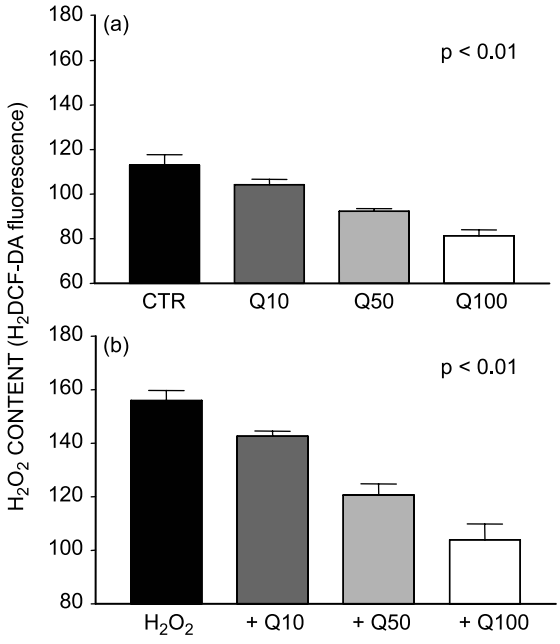

Figure 2. (a) H_2O_2 basal levels were measured by H_2DCF-DA staining in U937 incubated in absence (CTR) or in presence of QU 10, 50, 100 μM for 2 h. (b) H_2O_2 was added to the cultures during the last 15 min after a pre-incubating in absence (CTR) or in presence of QU 10, 50, 100 μM for 2 h. Results were expressed as median of fluorescence intensity (linearized values) ± S.E.M. The value of *p* has been obtained by two-way ANOVA test, considering all samples.

apoptotic, necrotic and viable cells. For this purpose, cells were stained simultaneously by different probes: Hoechst 33342, H_2DCF-DA, PI and ANX-V. As shown in Figure 3, fluorescence signals were analyzed after eliminating cell debris by applying the region depicted in the inserts present in panels a and b. Inserts show cell physical parameters, i.e. forward scatter (FSC) vs side scatter (SSC), the former referring to cell volume and the latter to cell complexity/density. Then, we have drawn quadrants on ANX-V *vs*. PI dot plot (panels a and b) to discriminate viable (ANX-V^- and PI^- cells, present in the lower left quadrant, painted in green), early apoptotic (ANX-V^+ and PI^- cells, lower right quadrant, red) and late apoptotic cells (ANX-V^+ and PI^+ cells, upper right quadrant, blue). These three populations were also discriminated by physical parameters. Viable cells were characterized by low SSC and high FSC, corresponding to cells with normal morphology; early apoptotic cells, by high SSC and low FSC; late apoptotic cells, by the highest SSC. It is noteworthy that the incubation with QU *per se* for 12 h provoked an increase of apoptotic and dead cells.

We then focused our attention on the analysis of viable cells (i.e. those colored in green). In the presence of QU 100 μM, these cells showed a decrease in H_2O_2 content (note the shift to the left of H_2DCF-DA fluorescence in panel d if compared with panel c; numbers indicate the linearized median fluorescence values). Moreover, most cells with a decreased H_2DCF-DA fluorescence did not exhibit markers of apoptosis (indeed, they were $AnxV^-$, see panel f *vs.* panel e), and showed a normal DNA content (panel h vs. panel g). The same results were observed at different times of incubation: 4, 6, 12, 16 h and for the doses 10 and 50 μM (data not shown). Thus, QU induces cell death in a given amount of cells (those red and blue), but has an anti-oxidant effect on the majority of cells, that are viable (green).

QU decreases GSH and H_2O_2 content, but increases O_2^- content

We analyzed the relationship between GSH, O_2^- and H_2O_2 content in cells exposed to QU. A staining with MBB, H_2DCF-DA and HE was then performed in cells treated for 6 or 12 h with 100 μM QU (Figure 4). Panels a–c show control, untreated cells; in all panels of this figure different colors were assigned to cells with normal (red), low (blue) or extremely low (green) H_2O_2 content. As described before, the treatment with QU for 6 h provoked a relevant decrease in H_2O_2 content (panel d). This phenomenon was accompanied by an increase in the number of cells with a reduced GSH content (panel e). After 6 h of treatment, in comparison with control cells (panel b), those with a decreased H_2O_2 content also displayed a diminished content of O_2^- (panel e). Interestingly, the population with intermediate H_2O_2 content started to change physical parameters (panel f, blue cells). After 12 h of incubation with QU, cells with intermediate H_2O_2 and low GSH content increase the production of O_2^- (panel h), and assumed the physical characteristics typical of apoptotic cells (panel i).

QU provokes a collapse of mitochondrial membrane potential ($\Delta\psi_m$) in living cells

We finally analyzed the relationship between GSH content and $\Delta\psi_m$ in living cells, i.e. those with the plasma membrane intact, that does not allow the entrance of PI into the cell (Figure 5). Panels a–c show control cells. In panel a, viable cells are painted in blue, those that incorporated low amount of PI in red (these cells are typically ANX-V^+, i.e. early apoptotic, and are well visible in Figure 3b, lower right quadrant. Note that, for technical reasons, in these experiments the amplification of the photomultiplier for the collection of the fluorescence signal from PI was set at a different level, and thus in Figure 5 cells with low PI incorporation are much brighter than those in Figure 3). The population of cells that assumed high amount of PI, i.e. those frankly necrotic or cellular debris, are painted in green (well visible in panel g). Untreated cells had high $\Delta\psi_m$ (in blue, panel b) and high GSH content (in blue, panel c); note that cells with low $\Delta\psi_m$ had also reduced GSH content (panel c).

Treating cells for 6 h with 100 μM QU did not provoke a dramatic increase in PI^+ cells (apoptotic or necrotic, panel d), but resulted in the onset of a population with intermediate $\Delta\psi_m$ (panel e, blue arrow); all living cells also displayed a reduced GSH content (note the shift to the left in panel f). Among them, those with intermediate $\Delta\psi_m$ had a further decrease in GSH (panel f, blue arrow). It is noteworthy that, in living cells, the decrease in GSH content occurred in cells with still a high $\Delta\psi_m$ (panel f, red arrow).

Incubation with QU for 12 h resulted in a significant increase in PI^+ cells, either apoptotic or necrotic (panel g), as already observed and reported in detail in Figure 3b. Living cells displayed a consistent decrease in $\Delta\psi_m$ (note the lowering of blue cells in panel h), along with a significantly reduced GSH content (panel i).

Discussion

Quercetin has been widely analyzed because of its multiple effects: antiallergenic, anti-inflammatory, anti-proliferative, anti-viral and anti-oxidant [12,40]. The mechanisms at the basis of these actions are still unclear. In particular, the effect of QU on cell oxidative balance reveals contradictory

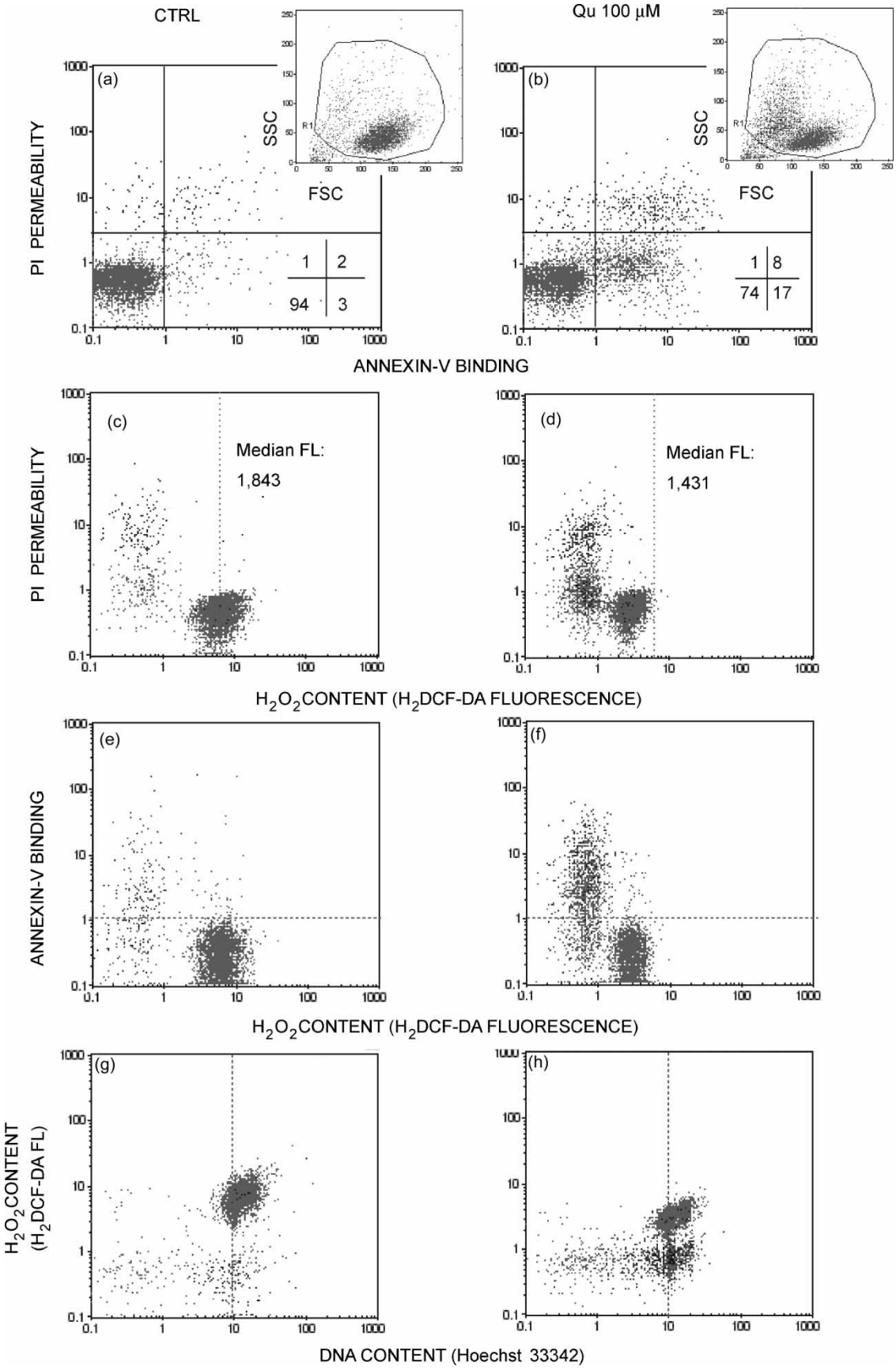

Figure 3. Cytofluorimetric analysis of U937 stained with ANX-V and PI (a, b); H_2DCFDA and PI (c, d); H_2DCF-DA and ANX-V (e, f); H_2DCF-DA and Hoechst 33342 (g, h). Cells were incubated in absence (CTRL, left column) or in presence of QU 100 µM (right column) for 12 h. Physical parameters (FSC *vs* SSC) are shown in the inserts of panels a and b. The figure is representative of three separate experiments.

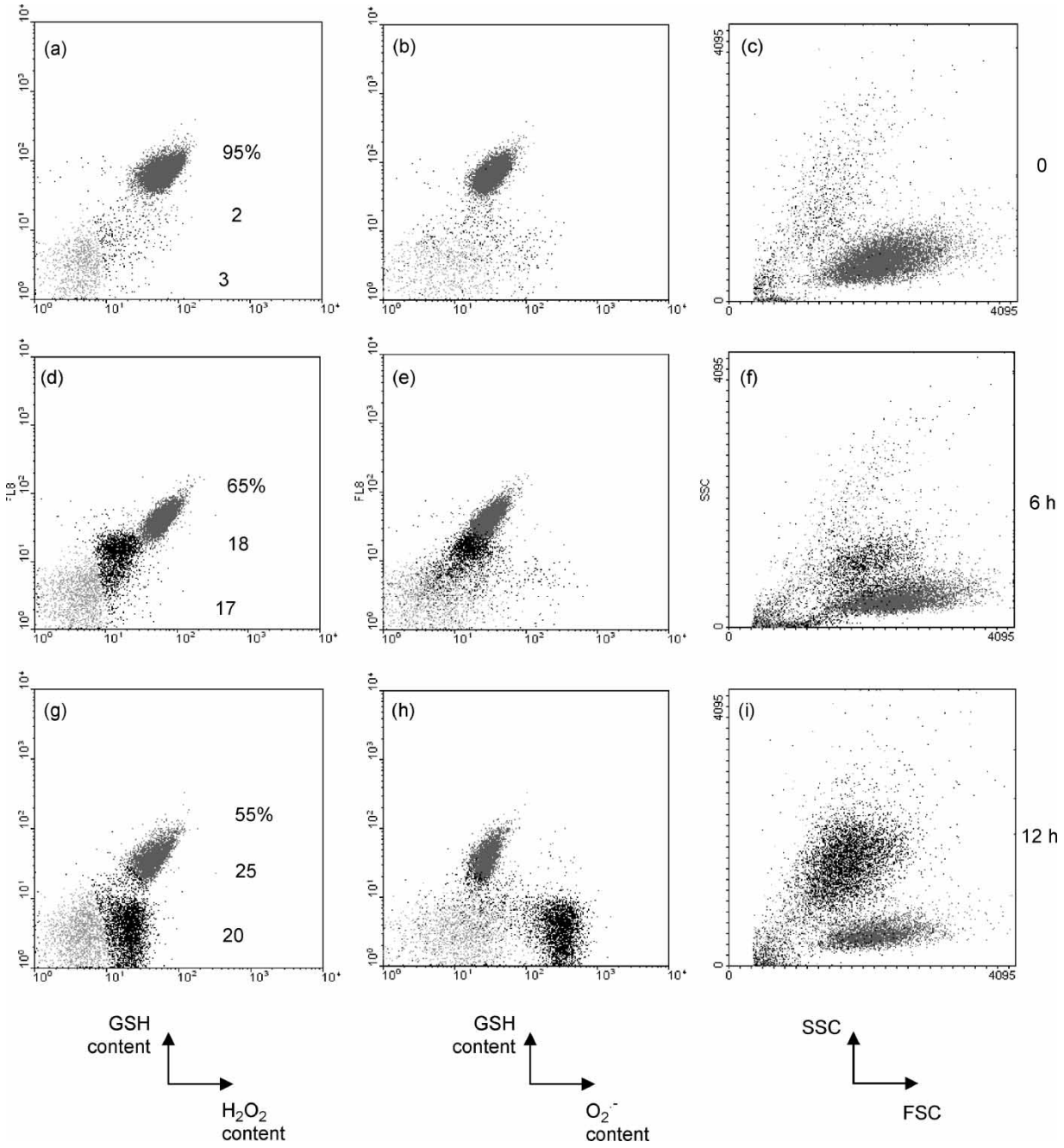

Figure 4. Cytofluorimetric analysis of U937 cells incubated for 6 and 12 h with 100 μM QU. Dot plots represent GSH *vs* H_2O_2 content (left column, panels a, d and g), GSH and O_2^- content (middle column, panels b, e and h) and physical parameters SSC *vs* FSC (right column, panels c, f and i). For details, see text in "Results". Numbers indicate the percentages of cells. The figure is representative of three separate experiments.

results [41–43]. The polyphenolic structure of QU allows scavenging of free radicals, and when QU reacts with ROS, potentially harmful oxidation products are formed [19]. Moreover, ROS have a double effect: if they are over-produced and inefficiently scavenged, they can damage several macromolecules. However, they can act as signaling molecules of great importance for signal transduction [4,5]. Mitochondria have a primary role in ROS production, because part of oxygen used in the respiratory chain is converted to free radicals that cause several injuries to these organelles [7]. Antioxidants are part of a complex network where scavengers, enzymes and vitamins modulate oxidative balance in cell.

The aim of the present study was to understand, at least in part, the role of QU in the anti/pro-oxidant network. We have used an original approach that utilizes polychromatic flow cytometry supported by excitation with multiple sources (two lasers and one UV lamp), and have been able to show

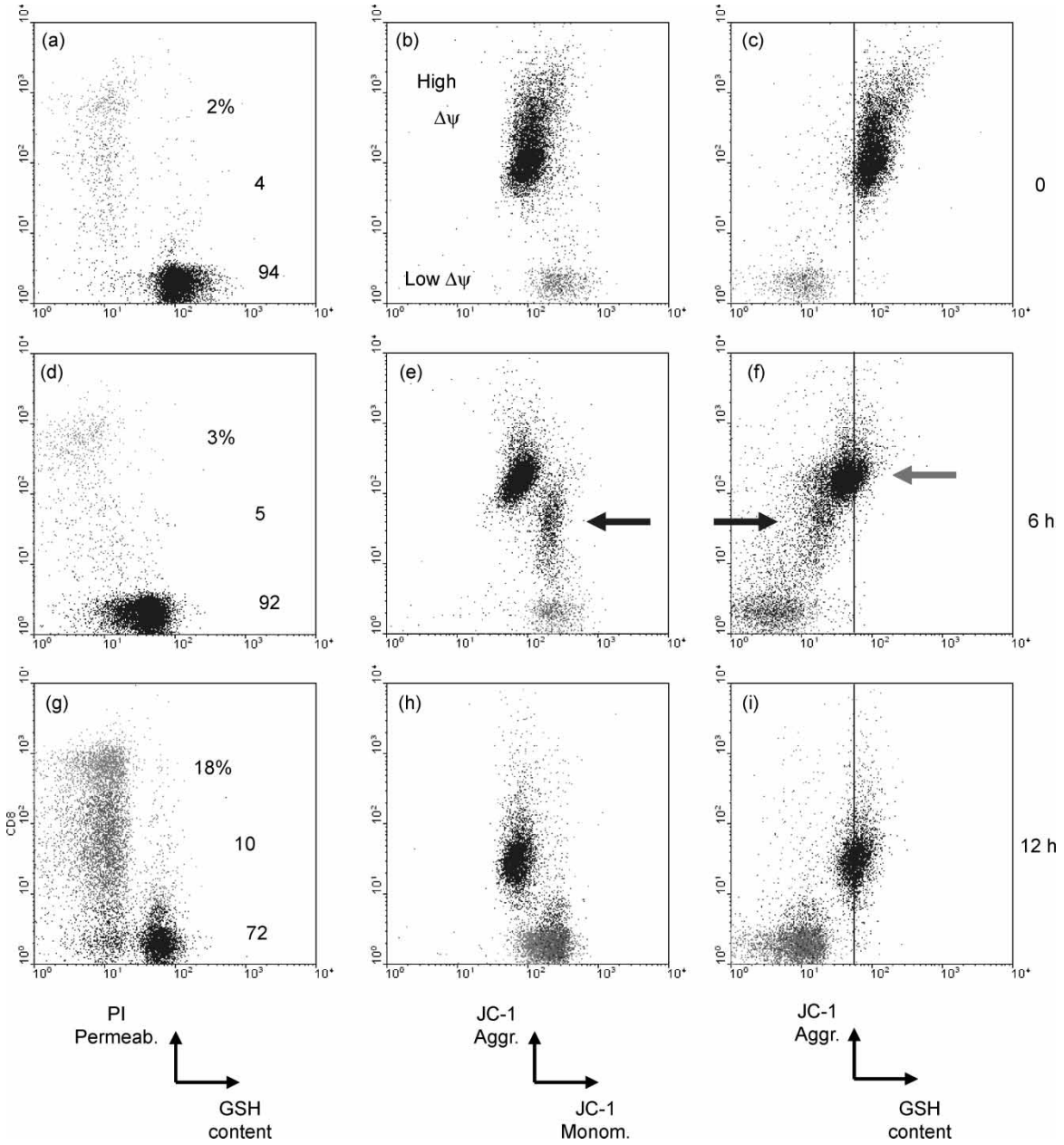

Figure 5. Cytofluorimetric analysis of U937 cells incubated for 6 and 12 h with 100 μM QU. Dot plots represent permeability to propidium iodide and GSH content (left column, panels a, d and g), mitochondrial membrane potential, i.e. JC-1 monomers and JC-1 aggregates (middle column, panels b, e and h) and JC-1 aggregates and GSH content (right column, panels c, f and i). For details, see text in "Results". Numbers indicate the percentages of cells. The figure is representative of three separate experiments.

simultaneously, for the first time, several parameters and activities in the same cell. Indeed, we could analyze GSH content, hydrogen peroxide and superoxide content, mitochondrial membrane potential, early and late apoptosis and cell viability. This approach is particularly significant since now different functional parameters linked to oxidative metabolism can now be analyzed in intact and living cells.

In order to investigate the effects of QU on ROS production, and the correlation with mitochondria membrane potential and cell viability, we have tested different doses and periods of exposure to the drug. As reported in previous studies [20,21], QU exerts an apoptotic action, which contrasts with its well-known anti-oxidant effect. The simultaneous analysis of H_2O_2, O_2^-, GSH and $\Delta\psi_m$ helped us in clarifying the role of QU on the cell oxidative balance. High doses of QU (50 and 100 μM) caused a decrease in H_2O_2 levels after 2 h of treatment; this effect was also maintained for long periods of incubation (until 24 h). While QU 10 μM slightly influenced H_2O_2 production for short times, for longer exposures the effect on H_2O_2 levels was similar to that observed for the higher doses.

The activity of QU as H_2O_2 scavenger was confirmed when H_2O_2 was added to cell cultures. The reduction in H_2O_2 levels indicated a minor exposure to this radical in cells and therefore, QU probably exerted an anti-oxidant effect, which was more effective for high doses. The simultaneous analysis of H_2O_2 content, PS exposition, DNA content and plasma membrane integrity ensures that reduced fluorescence of H_2DCF-DA observed in treated cells was due to a real antioxidant action of QU and not to its toxicity. However, as also demonstrated in other studies [19], QU exhibited simultaneously an apoptotic action on cell culture for prolonged exposure times.

Long time of exposure to QU resulted in a reduction in GSH content, suggesting the inability of QU to cope with ROS for such period. As previously reported, reacting with ROS QU can form potentially harmful oxidation products, which are inactivated by different molecules, preferentially reduced glutathione. Thus we propose that QU can exert an anti-oxidant effect in the presence of high levels of GSH. However, long exposure to QU results in a consumption of GSH and, as a consequence, reactive radical intermediates of QU could not be scavenged anymore. As a consequence, the pro-oxidant effect of this molecule could prevail over the antioxidant effect. In fact, after 12 h of incubation with QU 100 μM we found two cell populations with different O_2^- content, and cells with higher O_2^- content showed a massive GSH depletion. Moreover, these cells had the physical characteristic of apoptotic cells. In cells treated with QU 100 μM, the simultaneous analysis of H_2O_2 and O_2^- showed that the anti-oxidant activity of QU for short time (6 h) affected more intracellular content of H_2O_2 than of O_2^-. When cells were treated with QU for a long period, they showed an increase in O_2^-, a simultaneous loss in GSH content, and a total mitochondrial depolarization. Thus, it appears that prolonged or strong treatment with QU induces depletion of GSH stores. As a consequence, ROS are no longer scavenged and the pro-oxidant effect prevails on the anti-oxidant effect.

In conclusion, QU seems to exhibit a double action, anti- and/or pro-oxidant, which depends on cell oxidative balance and GSH content. Probably, when anti-oxidant system becomes inefficient, the consequent over production of ROS alters the cell redox state, and apoptotic program is activated. The polychromatic flow cytometric approach, which has been used to obtain the data presented here, is likely the best technology for understanding and better clarifying at the single cell level the complex relationship among anti-proliferative, pro-apoptotic, anti- and pro-oxidant effects of QU as well as of other molecules.

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