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Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid☆

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Abstract

Quercetin uptake in Jurkat cells is extremely rapid and associated with a remarkable accumulation of the flavonoid, dependent on its binding to intracellular components. Cell-associated quercetin is biologically active, quantitatively consumed to promote survival in the presence of reactive species, such as peroxynitrite ($ONOO^-$), or reduction of extracellular oxidants via activation of plasma membrane oxidoreductases. In alternative, quercetin is very slowly released upon post-incubation in drug-free medium, an event significantly accelerated by extracellular albumin. Quercetin uptake is also observed in isolated mitochondria, resulting in an enormous accumulation of the flavonoid, consumed under conditions associated with prevention of lipid peroxidation induced by $ONOO^-$. Interestingly, remarkable quercetin accumulation is also detected in the mitochondria isolated from quercetin-pre-loaded cells, and exposure to either $ONOO^-$ or extracellular oxidants caused the parallel loss of both the mitochondrial and cytosolic fractions of the flavonoid. In conclusion, Jurkat cells accumulate large amounts of quercetin and even larger amounts of the flavonoid further accumulate in their mitochondria. Intramitochondrial quercetin appears to be functional for prevention of mitochondrial damage as well as for redistribution to the cytosol, when the fraction of the flavonoid therein retained is progressively consumed either by cell-permeant oxidants or by activation of plasma membrane oxidoreductases.
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Keywords: Quercetin; Cellular uptake; Mitochondrial accumulation; Cytoprotection; Trans-plasma membrane oxidoreductase

1. Introduction

Quercetin is a naturally occurring flavonoid producing beneficial effects for the human health [1–3], via scavenging of reactive oxygen species/metal chelation and stimulation/inhibition of enzyme activities/signal transduction pathways [4–7]. While the hierarchy and relevance of these effects is not yet fully appreciated, it appears obvious that its accumulation in specific subcellular compartments might dictate the specificity of the effects mediated by the flavonoid. We can therefore predict that cells will benefit from the accumulation of quercetin in the mitochondrial compartment, central for both the onset of signaling pathways initiated by mitochondrially generated radical species [8] and for the triggering of events promoting death via release of apoptogenic factors [9–11].

We recently investigated quercetin uptake in red blood cells (RBC) and found that this event is extremely rapid and associated with the binding of the flavonoid to hemoglobin. Indeed, high concentrations of quercetin were almost quantitatively removed from the extracellular milieu by RBC (10% hematocrit) in less than 5 min [12]. We also determined that RBC release quercetin in the presence of albumin or serum and that quercetin pre-saturated albumin leads to accumulation of the flavonoid in naive RBC [12].

Quercetin efficiently crosses plasma membranes also in various mammalian cell types [13–15], a notion consistent with the chemical structure of the flavonoid [16]. In addition, preliminary evidence documents its ability to reach the mitochondrial compartment [17], and numerous studies report effects of quercetin on mitochondrial functions [18–21] that would be best explained by the mitochondrial accumulation of the flavonoid. As a final note, the remarkable content of heme-containing proteins should allow a significant mitochondrial accumulation of the flavonoid.

Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, cyanide-*p*-trifluoromethoxyphenylhydrazone; FIC, ferricyanide; FOC, ferrocyanide; GDH, glutamate dehydrogenase; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; RBC, red blood cells; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances.

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The hypothesis tested in this study was that intracellular quercetin may accumulate in the intramitochondrial compartment and eventually return to the cytosol upon oxidation of the cytosolic/plasma membrane fraction of the flavonoid. Our results were consistent with this notion, thereby suggesting that the high mitochondrial content of quercetin is functional to both the scavenging of reactive oxygen species, extensively generated in this compartment, and redistribution on demand, that is, when its cytosolic fraction is depleted.

2. Materials and methods

2.1. Chemicals

Quercetin, 1,10-phenanthroline, Na-acetate, ethyl acetate, Triton X-100, dithiothreitol (DTT), Hepes, ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), digitonin, ethylenediaminetetraacetic acid, Tris–HCl as well most reagent-grade chemicals were purchased from Sigma-Aldrich (Milan, Italy). $K_3Fe(CN)_6$, $FeCl_3$, citric acid, dimethyl sulfoxide (DMSO), NaH_2PO_4 and acetonitrile were Carlo Erba products (Milan, Italy). RPMI 1640, fetal bovine serum and penicillin–streptomycin were obtained from CELBIO (Milan, Italy). MitoTracker Red CMXRos was from Molecular Probes (Leiden, The Netherlands).

2.2. Cell culture and treatment conditions

Jurkat human T lymphoblast cells were cultured in suspension in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C gassed with an atmosphere of 95% air–5% CO_2.

Treatments with quercetin (1×10^6 cells/ml) were performed in prewarmed saline A (8.182 g/L NaCl; 0.372 g/L KCl; 0.336 g/L $NaHCO_3$; 0.9 g/L glucose, pH 7.4). A stock solution of quercetin (20 mM) was prepared in DMSO and diluted in saline A before use. The final DMSO concentration was never greater than 0.5% and, under these conditions, DMSO was neither toxic nor did it affect the cytotoxic properties of peroxynitrite ($ONOO^-$). Peroxynitrite was synthesized by the reaction of nitrite with acidified H_2O_2 as described in Ref. [22], with minor modifications [23]. MnO_2 (1 mg/ml) was added to the mixture for 30 min at 4°C to eliminate the excess of H_2O_2. MnO_2 was removed by centrifugation and filtration through 0.45-μm-pore microfilters. The solution was frozen at −80°C for 24 h. The concentration of peroxynitrite, which forms a yellow top layer due to freeze fractionation, was determined spectrophotometrically by measuring the absorbance at 302 nm in 1.5 M NaOH (ε_{302}=1670 M^{-1} cm^{-1}). Treatments with $ONOO^-$ were performed in prewarmed saline A containing 2.5×10^5 cells/ml.

2.3. Measurement of quercetin content

After quercetin exposure, the cells were washed with saline A, resuspended in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and lysed with a sonicator. Quercetin was then extracted with ethyl acetate, and these samples were first taken to dryness by rotary evaporation, subsequently redissolved in DMSO and finally diluted with bi-distilled water just before high-performance liquid chromatography (HPLC) analysis (λ max 370 nm). The same assay was performed in ethyl acetate extracts (processed as detailed above) obtained from the extracellular milieu (prior to and after quercetin exposure) and from the mitochondria of cells previously exposed to quercetin. In other experiments, mitochondria were first isolated from untreated cells and then exposed to the flavonoid prior to ethyl acetate extraction, processing of the samples and HPLC analysis.

HPLC analysis was performed as described by Day et al. [24], with minor modifications. The assay involved the use of a 25 cm×4.6 mm Discovery C-18, 5-μm column (Supelco, Bellefonte, PA) equipped with a Supelguard Discovery C-18 guard column (2 cm×4 mm, 5 μm). Solvents A (0.1% formic acid) and B (acetonitrile) were run at a flow rate of 1 ml/min. The running gradient was adjusted to 17% B (2 min), increasing to 25% B (5 min), 35% B (8 min), 50% B (5 min) and then 100% B (10 min), followed by a reequilibration at 10% B (15 min). All solvents were HPLC grade (Aldrich-Sigma), and water was purified via a Milex Q-plus system (Millipore).

Quercetin concentrations were determined from the corresponding calibration curve constructed with the pure chemical dissolved in ethyl acetate. Intracellular and mitochondrial concentrations of quercetin were calculated using published values for cell [25] and mitochondrial [26,27] volume, respectively.

2.4. Purification of mitochondria

Cells (25×10^6) were suspended in ice-cold buffer A (5 mM Hepes; 210 mM mannitol; 70 mM sucrose; 1 mM EGTA; pH 7.4) containing 10 μg/ml digitonin and mechanically homogenized with a glass potter. The homogenate was centrifuged at 750×*g* for 20 min at 4°C to pellet cell debris and generate a crude cytoplasmic fraction. Mitochondria were then pelleted by centrifuging the cytoplasmic fraction at 10,000×*g* for 30 min at 4°C [28].

The activities of lactate dehydrogenase (LDH, a cytosolic marker) and glutamate dehydrogenase (GDH, as mitochondrial marker) were determined [29] to assess the purity of the mitochondrial fraction. Typically, there was a difference of about two orders of magnitude in LDH activities detected in the cell and mitochondrial homogenates (e.g., 834.65 and 9.64 U/mg protein, respectively). Along the same lines, GDH activity was extremely lower in cell lysates than in mitochondrial homogenates (e.g., 0.425 and 246.2 U/mg protein, respectively). Mitochondrial O_2 consumption was measured as an index of mitochondrial function and integrity using a YSI oxygraph equipped with a Clark electrode (model 5300, Yellow Springs Instruments, Yellow Springs, OH, USA). The mitochondrial suspension (3 ml) was transferred to the polarographic cell, and the rate of oxygen utilization was monitored under constant stirring for 3 min (basal respiration). Cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was then added, and once again respiration was measured for 3 min. The rate of oxygen utilization was calculated as described previously [30]. Before and after FCCP addition, oxygen consumption was 54.0 and 210.3 nmol/min per milligram of protein, respectively.

Isolated mitochondria were exposed to quercetin in Buffer A and finally resuspended in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, pH 7.5) prior to ethyl acetate extraction and HPLC analysis, as described above. Mitochondria obtained from cells previously exposed to quercetin were also lysed and processed as indicated for isolated mitochondria.

2.5. Assessment of quercetin fluorescence

Cells were exposed for 10 min to 50 μM quercetin in 35-mm tissue culture dishes containing an uncoated coverslip. Subsequently, the cells were post-incubated for a further 10 min with 10 nM MitoTracker Red CMXRos. Under these conditions, Jurkat cells rapidly attach to the coverslip. After treatments, the cells were washed three times and analyzed with a fluorescence microscope (BX-51, Olympus Italia, Milan, Italy) equipped with a SPOT-RT camera unit (Diagnostic Instruments, Delta Sistemi, Rome, Italy). The excitation and emission wavelengths were 488 and 515 nm (quercetin) and 580 and 610 nm (MitoTracker Red CMXRos), and with a 55-nm slit width for both emission and excitation. Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition per experiment. Quercetin fluorescence was also determined in isolated mitochondria (0.25-mg proteins) preloaded with the flavonoid with or without subsequent exposure (10 min) to $ONOO^-$ and 3 μM $CaCl_2$ (added immediately prior to $ONOO^-$). The samples were then centrifuged and the pellets were first suspended in 200 μl of Buffer A and finally analyzed spectrofluorimetrically (λ_{ex} = 430 nm; λ_{em} = 595 nm) with a plate reader (Spectrafluor plus, TECAN, Austria).

2.6. Ferricyanide reduction assay

Ferricyanide (FIC) reduction was determined to obtain an estimate of transplasma membrane oxidoreductase activity, as reported by Avron and Shavit [31]. Briefly, the cells (1×10^6 cells/ml) were first exposed to quercetin, washed twice with saline A and incubated for a further 30 min in PBS (pH 7.4) containing 2.5 mM adenosine and 1 mM FIC (potassium salt), dissolved immediately prior to use. FIC is membrane impermeant and the transplasma membrane oxidoreductase promotes its reduction to ferrocyanide (FOC), determined using 1,10-phenanthroline as an indicator and measuring absorption at 510 nm (ε_{510} = 10,500 M^{-1} cm^{-1}).

2.7. Cytotoxicity assay

Cytotoxicity was determined with the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue and viable cells (i.e., those excluding trypan blue) were counted with a hemocytometer.

2.8. Lipid peroxidation assay

Quercetin-pre-loaded mitochondria (0.75-mg proteins), suspended in 1 ml of buffer A, were exposed for 10 min to 250 μM $ONOO^-$. Thiobarbituric acid-reactive substances (TBARS) were determined as described by Santos et al. [32]. Briefly, the mitochondrial suspension was mixed with 2 ml of thiobarbituric acid (TBA) medium, containing 250 mM HCl, 15% trichloroacetic acid, 3 mM TBA and 0.1% butylated hydroxytoluene. The color was allowed to develop in the dark for 12 h and the absorbance was measured at 533 nm. The amount of malondialdehyde was calculated from ε_{533} = 149,000 M^{-1} cm^{-1} [32].

2.9. Statistical analysis

Results are expressed as means±S.D. Statistical differences were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparison or two-way ANOVA followed by Bonferroni's test for multiple comparison. A value of $P<.05$ was considered significant.

3. Results

3.1. Quercetin accumulates in Jurkat cells and is therein retained in a biologically active form

Fig. 1A illustrates a representative chromatogram of a quercetin standard solution with a retention peak corresponding to that obtained after analysis of ethyl acetate extracts from Jurkat cells preexposed to the flavonoid (Fig. 1B). Using this method, we recovered 95% of the flavonoid in the initial solution (nominally 50 μM), whereas only a residual 85% was detected after a 10-min incubation of 1×10^6 cells in 1 ml of this medium (Fig. 1C). Under these conditions, 3.25 nmol of quercetin were associated with the cells, thereby suggesting a remarkable intracellular accumulation of the flavonoid (see below). The results illustrated in Fig. 1D document a linear relationship between the initial concentration and the fraction of the flavonoid associated with the cells.

Quercetin uptake was extremely rapid (Fig. 1E) and, after equilibrium was reached, quercetin release was rather slow since approximately 60% of the initial amount of the flavonoid was still

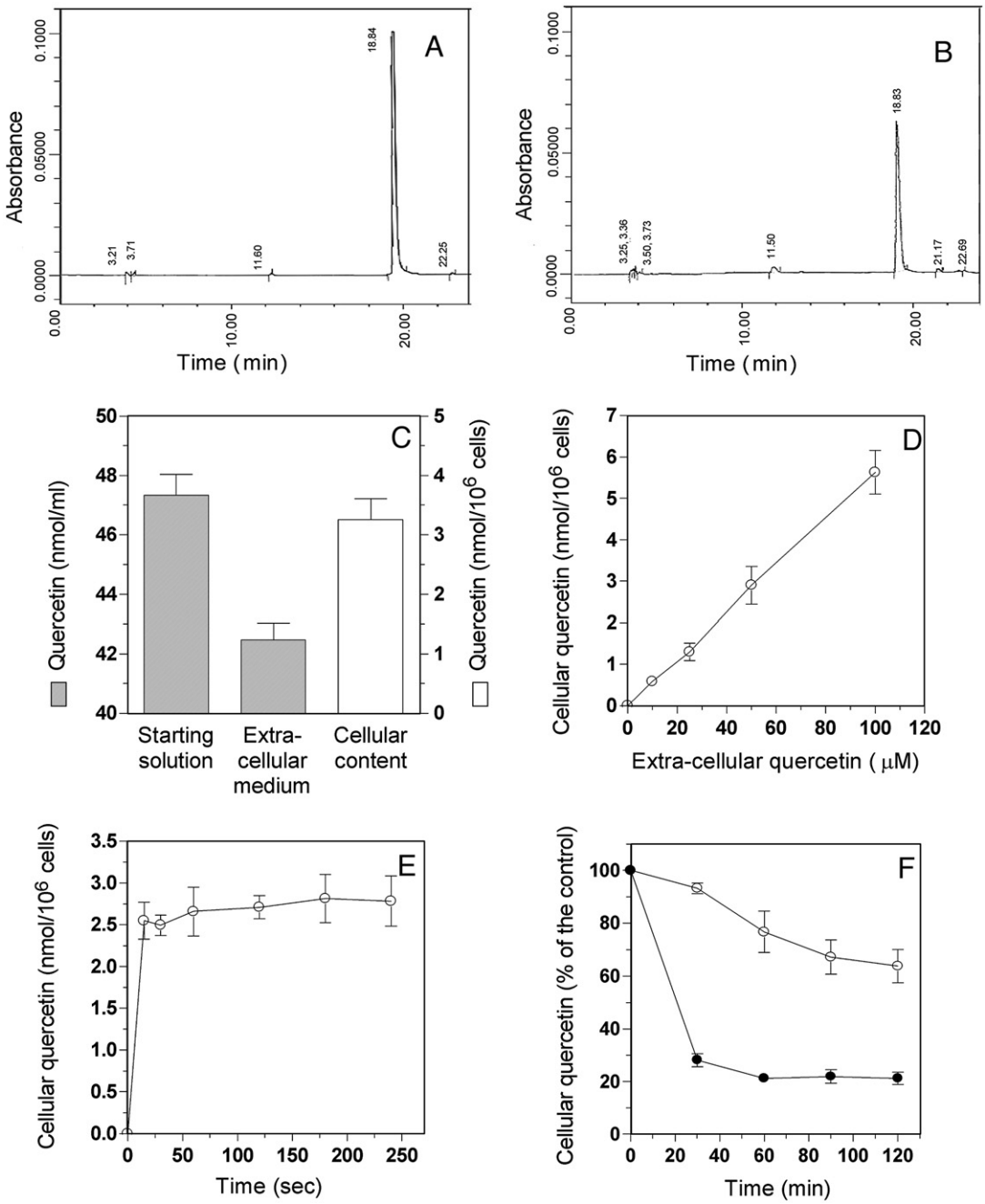

Fig. 1. Quercetin uptake in Jurkat cells. (A) Chromatogram of a quercetin standard solution (100 μM). (B) Chromatogram of an ethyl acetate extract of Jurkat cells previously exposed for 10 min to 50 μM quercetin. (C) Quercetin content of ethyl acetate extracts obtained from a saline containing 50 μM quercetin prior to and after a 10-min incubation in the presence of the cells. Ethyl acetate extracts of lysates obtained from these cells were also assayed for their quercetin content. (D) Quercetin content of cells exposed for 10 min to increasing concentrations of quercetin. (E) Time dependence of quercetin (50 μM) uptake and accumulation in intact cells; (F) quercetin release from quercetin-pre-loaded cells incubated in saline A in the absence (open circles) or presence (closed circles) of 25 mg/ml albumin. Results represent the means±S.D. calculated from at least three separate experiments.

associated with the cells after a 2-h post-incubation in fresh saline A (Fig. 1F). The rate of loss of intracellular quercetin was however dramatically accelerated upon addition of albumin to the extracellular milieu (Fig. 1F).

These results are consistent with the notion that quercetin is rapidly and effectively taken up by Jurkat cells. The remarkable intracellular accumulation of quercetin can only be explained by its binding to intracellular biomolecules, a conclusion also consistent with the notion that large amounts of unbound quercetin would redox cycle, thereby producing reactive species and the ensuing toxic events. Quercetin-pre-loaded cells however remained viable, as assessed by the trypan blue exclusion assay and other biochemical criteria (not shown). In addition, quercetin-pre-loading failed to promote delayed cell death and had hardly any effect on the rate of cell proliferation (not shown).

As a final note, quercetin-pre-loaded cells displayed enhanced oxygen consumption rates, a notion in keeping with the results obtained using purified mitochondria [20]. In particular, in cells preexposed to 50 μM quercetin, we detected an oxygen consumption rate of 1.78 nmol·min^{-1}×10^6 cells vs. the 1.56 nmol·min^{-1}×10^6 cells measured in untreated cells (14% increase). These results are therefore consistent with a significant mitochondrial accumulation of the flavonoid.

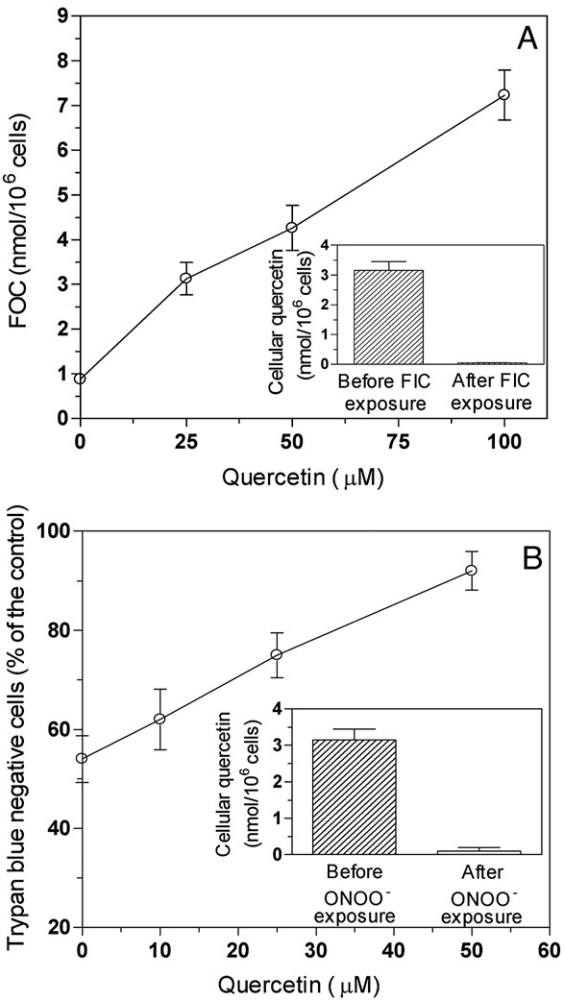

Fig. 2. Intracellular quercetin promotes reduction of an extracellular oxidant and suppresses toxicity induced by ONOO$^-$. The cells were preloaded (10 min) with increasing concentrations of quercetin and treated either for 30 min with 1 mM FIC (A) or for 60 min with 200 μM ONOO$^-$ (B). After treatments, FOC formation was assayed in the supernatant (A) and cytotoxicity immediately determined with the trypan blue exclusion assay (B), as described in Materials and methods. Quercetin levels were determined before and after exposure to either FIC (inset to Panel A) or ONOO$^-$ (inset to Panel B). Results represent the means±S.D. calculated from at least three separate experiments.

Intracellular quercetin is biologically active since able to remarkably enhance reduction of an extracellular oxidant (1 mM FIC, Fig. 2A), an event mediated by the activity of a transplasma membrane oxidoreductase. This notion is documented by our previous studies ruling out the possibility of direct FIC reduction by released quercetin [12,15,16,33] and further supported by several observations. (i) FOC formation was sensitive to transplasma membrane oxidoreductase inhibitors (not shown). (ii) There was no evidence of significant quercetin release (Fig. 1F) during the time interval (30 min) employed in the FIC reduction assay. (iii) There was no evidence of FOC formation after incubation of FIC in the same medium in which quercetin-pre-loaded cells had been grown for 30 min (not shown). The analysis in Fig. 2A also provides the important information that the net amount of FOC generated by quercetin (50 μM) pre-loaded cells (i.e., about 3 nmol/10^6 cells) corresponds to the nmol of flavonoid consumed (inset). These results also argue against the possibility of intracellular quercetin redox cycling.

Fig. 2B shows an additional important effect mediated by intracellular quercetin, that is, suppression of toxicity induced by a powerful direct oxidant, ONOO$^-$ (200 μM). This response was also associated with loss of detectable intracellular quercetin (inset).

Taken together, the results presented in this section indicate that quercetin is taken up by passive diffusion and binds to some intracellular component so that large amounts of the flavonoid accumulate in a very short time. Quercetin is retained by the cells in a biologically active form and can be quantitatively consumed upon intra/extracellular oxidant supplementation.

3.2. Quercetin accumulates in Jurkat cell mitochondria

Quercetin uptake was investigated in isolated mitochondria exposed to the same quercetin concentrations employed with intact cells (i.e., 10–100 μM). Data illustrated in Fig. 3A provide the putative intramitochondrial concentrations, based on aqueous volumes of these organelles, in comparison with the concentrations detected in intact cells (shown in Fig. 1D), normalized to cellular aqueous volumes. It was interesting to observe that mitochondria accumulate remarkably greater concentrations of the flavonoid than intact cells. As an example, exposure to 50 μM quercetin led to intramitochondrial levels as high as 100 mM in comparison to the 40-fold lower concentration detected in intact cells.

The above considerations are obviously based on apparent quercetin concentrations (a considerable fraction of the flavonoid is most likely imbedded in lipid membranes or bound to other intracellular molecules) but nevertheless clearly emphasize the existence of an excess of intramitochondrial quercetin-binding molecules.

As indicated in Fig. 3B, there was a remarkable mitochondrial accumulation of the flavonoid also after exposure of intact cells to 10–100 μM quercetin. The same linear relationship is reported in the inset of this figure, showing a plot of the mitochondrial vs. the cellular concentrations of the flavonoid. When calculated under conditions of exposure to 50 μM quercetin, cellular concentration was close to 3 mM, whereas mitochondrial quercetin approached the apparent concentration of 35 mM. Fig. 3C provides the additional information that the net amount of quercetin accumulating in mitochondria corresponds to about 11% of the cell-associated fraction of the flavonoid, regardless of its initial extracellular concentration.

These results therefore lead to the univocal conclusion that quercetin accumulates in cells and subsequently accumulates even further in mitochondria. This nonhomogenous intracellular distribu-

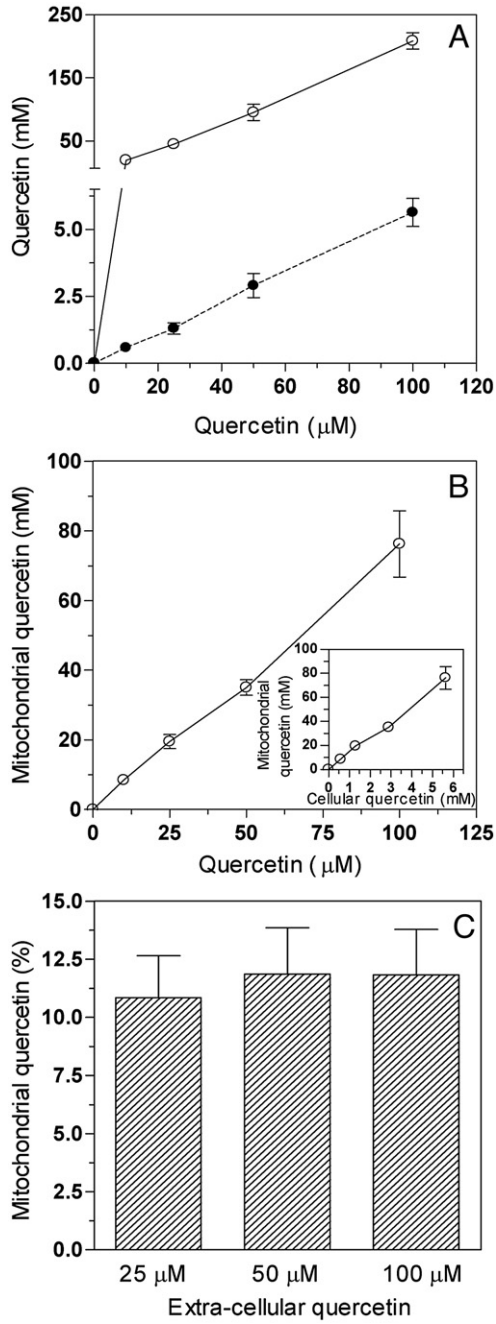

Fig. 3. Quercetin accumulation in Jurkat cell mitochondria. (A) Mitochondria were first purified (0.15-mg proteins) and subsequently incubated for 10 min with increasing concentrations of quercetin (open circles). Quercetin content was then assayed in ethyl acetate extracts obtained from the mitochondrial preparations and the results normalized to mitochondrial volumes. Also shown are the results obtained using intact cells (closed circles, data from Fig. 1D). (B) Cells were exposed for 10 min to increasing concentrations of quercetin and immediately processed for the isolation of mitochondria in which the content of the flavonoid was determined as detailed above. Mitochondrial levels of quercetin are plotted against the extracellular (main graph) and intracellular (inset) concentrations of quercetin. (C) Cells were treated for 10 min with 25–100 μM quercetin and processed to assess cell-associated and mitochondrial contents of the flavonoid. In order to obtain an indication on the relative distribution in these compartments, the results are normalized with respect to cell number. Results represent the means±S.D. calculated from at least three separate experiments.

tion of quercetin implies that quercetin-binding molecules are more densely expressed within mitochondria than in the cytosol and/or other organelles.

3.3. Mitochondrial effects of quercetin

Since mitochondria accumulate very large amounts of quercetin, it is important to know whether the flavonoid is stored by these organelles in a biologically active form and, if this is the case, whether it can be released and exert its functions in other compartments. A preliminary indication of biological activity is provided by the enhanced oxygen consumption rates observed in cells preloaded with the flavonoid (see above).

We next investigated the impact of mitochondrial quercetin on the deleterious effects mediated by $ONOO^-$. For this purpose, mitochondrial preparations were exposed to $ONOO^-$ (250 μM)/$CaCl_2$ (3 μM), with or without prior quercetin preloading, and then assayed for TBARS formation, an index of membrane lipid peroxidation. It was found that quercetin suppresses TBARS formation elicited by $ONOO^-$ (Fig. 4A), an event accompanied by a significant loss of mitochondrial quercetin (Fig. 4B).

These results indicate that mitochondrial quercetin is biologically active. In addition, these findings, along with those showing quantitative depletion of the cellular flavonoid after exposure to a membrane impermeant oxidant (inset to Fig. 2A), under conditions of extensive reduction of the latter (Fig. 2A), would also suggest

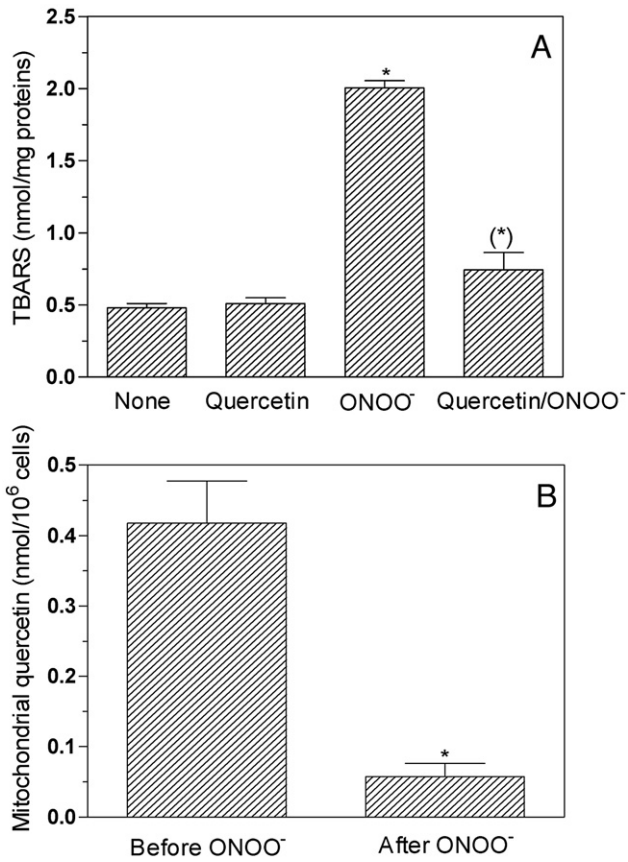

Fig. 4. Quercetin prevents lipid peroxidation induced by $ONOO^-$ in isolated mitochondria. (A) Mitochondria (0.75 mg of proteins), isolated from cells exposed for 10 min to 0 or 50 μM quercetin, were treated for 10 min with 0 or 250 μM $ONOO^-$/3 μM $CaCl_2$. $CaCl_2$ was added to the mitochondrial preparations immediately prior to $ONOO^-$. TBARS were then determined as described in Materials and methods. *P≤.001 compared with untreated mitochondria; (*) P≤.001 compared with mitochondria exposed to $ONOO^-$ (one-way ANOVA followed by Dunnett's test). (B) Quercetin levels were determined in the mitochondria isolated from quercetin-pre-loaded cells before and after exposure to $ONOO^-$/$CaCl_2$. Results represent the means±S.D. calculated from three separate experiments. *P≤.001 compared with mitochondria prior to $ONOO^-$ exposure.

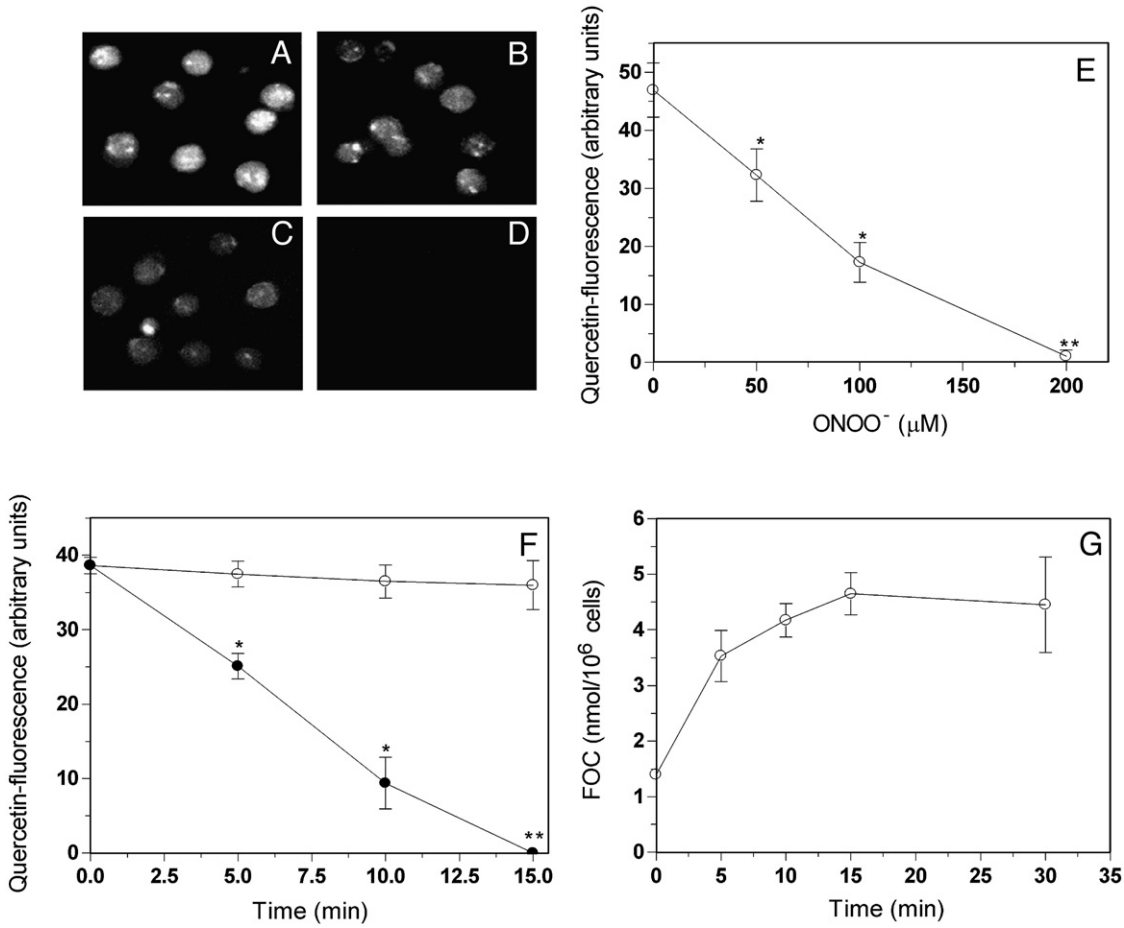

Fig. 5. Quercetin oxidation determined by measuring quercetin fluorescence. (A–D) Representative microscope images of quercetin (50 μM) pre-loaded (10 min) cells after exposure (10 min) to 0 (A), 50 (B), 100 (C) or 200 (D) μM $ONOO^-$. Quantitative analysis of total fluorescence (both punctate and diffused) produced the results shown in E. (F) Quercetin-pre-loaded cells were incubated for increasing time intervals in the absence (open circles) or presence (closed circles) of FIC (1 mM) and immediately analyzed for quercetin fluorescence, as detailed above. (G) Time dependence of FOC formation by quercetin-pre-loaded cells. Cells were exposed for 10 min to 50 μM quercetin, and extracellular FOC formation was determined at increasing time intervals after FIC addition. Results represent the means±S.D. calculated from three separate experiments. $^*P<.01$; $^{**}P<.001$ compared with the fluorescence from cells that did not receive $ONOO^-$ or FIC (two-way ANOVA followed by Bonferroni's test).

redistribution of mitochondrial quercetin to the cytosolic/plasma membrane compartments. FIC reduction is indeed mediated by a plasma membrane oxidoreductase [34–38].

We employed an additional strategy to prove the occurrence of mitochondrial quercetin accumulation and redistribution in intact cells, taking advantage of the ability of the flavonoid to emit fluorescence in living cells [39]. We reasoned that quercetin accumulation in the mitochondria should promote a punctate fluorescence, distinguishable from a background fluorescence due to quercetin binding to cytosolic components. The image obtained (Fig. 5A) is in keeping with this premise. Interestingly, exposure to increasing concentrations of $ONOO^-$ (Fig. 5B–D) had an impact on both the punctate and diffused fluorescence. The dose-response, calculated from more than 50 cells/experiment in three different experiments (Fig. 5E), provides evidence of loss of total fluorescence after exposure to 200 μM $ONOO^-$. The correspondence of the punctate quercetin fluorescence with the mitochondrial compartment was confirmed by the observation that this signal colocalizes with the fluorescence emitted by a mitochondrial probe (not shown).

These results provide indirect evidence of biological activity of mitochondrial quercetin but however do not prove cytosolic replenishment from mitochondrial quercetin. In order to obtain results in this direction, we decided once again to perform experiments measuring transplasma membrane oxidoreductase activity. We previously showed in RBC that electrons are provided by the fraction of quercetin associated with the plasma membrane, in equilibrium with the fraction of the flavonoid associated with cytosolic components, in particular hemoglobin [12]. This means that, in the presence of extracellular oxidants, a dynamic flux of quercetin released by hemoglobin occurs in parallel with quercetin oxidation in the plasma membrane, so that quercetin can be eventually quantitatively consumed. We predicted the existence in our cells of a similar equilibrium, however, involving a third compartment (i.e., mitochondria). The results obtained were indeed in keeping with this notion, since addition of FIC to quercetin-pre-loaded cells caused time-dependent loss of quercetin fluorescence (Fig. 5F) in parallel with the appearance of FOC (Fig. 5G). It was interesting to observe that complete loss of detectable quercetin fluorescence is detected at the same time point (15 min) in which FOC formation reaches a plateau. The images obtained in studies measuring quercetin fluorescence, not shown here for the sake of brevity, were virtually identical to those reported in Fig. 5A–D. Time-dependent loss of both the punctate and diffused fluorescence was clearly detected. Under the same conditions, there was no apparent loss of quercetin fluorescence in cells incubated without FIC (Fig. 5F).

Taken together, these results emphasize the notion that mitochondrial quercetin is biologically active and may redistribute to the cytosol and/or plasma membrane when the equilibrium is altered by quercetin consumption in these compartments.

4. Discussion

The present study provides results indicating that quercetin efficiently crosses Jurkat cell plasma membranes, leading within seconds to the accumulation of very large amounts of the flavonoid (Fig. 1). Intracellular accumulation of the flavonoid was indeed impressive since approximately 7% of the initial extracellular quercetin was associated with the cells, in a volume about 3 orders of magnitude smaller.

These results, along with the observation that quercetin is effectively retained upon post-incubation, unless albumin was added to the extracellular milieu, imply that cellular uptake is mediated by passive diffusion. It is important to note that quercetin avidly binds to albumin because of the multiplicity of binding sites provided by phenolic groups (hydrogen bonds) and by sites for hydrophobic interaction [12,40]. The notion that quercetin readily penetrates the plasma membrane by passive diffusion and eventually accumulates intracellularly therefore demonstrates the existence of a redundancy of binding sites, unsaturated even at very high quercetin concentrations. There was indeed a linear accumulation of the flavonoid after exposure to 10–100 μM quercetin, resulting in hypothetical (i.e., calculated assuming that the entire fraction of the cell-associated quercetin was unbound) millimolar levels.

We immediately thought that heme-containing proteins were potentially important quercetin-binding molecules since our previous work performed in RBC provided evidence of remarkable accumulation dependent on extensive binding of the flavonoid to hemoglobin [12,40–42]. Quercetin binding to hemoglobin was recently documented by fluorescence quenching as well as by absorption and circular dichroism spectroscopy [43]. More generally, however, the ability of quercetin to bind heme-containing proteins is strongly suggested by numerous studies documenting effects (stimulation or, more frequently, inhibition) of Phase I monoxygenase enzyme activities (for a review, see Ref. [44]). Obviously, quercetin can also bind to a variety of additional molecules because of the above-mentioned multiplicity of sites allowing hydrogen bonds and hydrophobic interaction. Thus, it is plausible that quercetin binds to a variety of cytosolic proteins and accumulates in lipid compartments (i.e., membranes). In this perspective, hydrophobic compartments such as mitochondria, in which heme-containing proteins are largely expressed, may represent a site of preferential accumulation of the flavonoid.

The results obtained were in line with the above premise since isolated mitochondria were at least 40 times more effective than whole cells in accumulating the flavonoid (Fig. 3A). Interestingly, a similar, remarkable quercetin accumulation was measured in the mitochondria isolated from quercetin-pre-loaded cells (Fig. 3B). The linear relationship existing between the extracellular concentration (10–100 μM) and the mitochondrial accumulation of the flavonoid documents the existence within mitochondria of a very large number of quercetin-binding molecules (Fig. 3B). The same linear relationship was obtained by replacing extracellular quercetin with the cellular concentration of the flavonoid (inset to Fig. 3B). Consistent with the notion that mitochondria contain a large number of quercetin-binding molecules was also the observation that the fraction of intramitochondrial quercetin was always constant (i.e., 11% of cell-associated quercetin), regardless of the extracellular concentration of the flavonoid (Fig. 3C).

These findings demonstrate that a significant amount of cell-associated quercetin is stored within mitochondria, a conclusion bearing two different and important consequences. Mitochondria represent a critical source of reactive oxygen species and their ability to accumulate quercetin may be functional for protection of mitochondrial function and integrity. Quercetin indeed displays iron chelating [4,6,45] and antioxidant [5,6] properties and modulates/interferes with a large variety of enzyme activities/signal transduction pathways [7]. The ability of quercetin to prevent cell death associated with a variety of toxicity paradigms is very well established and consistent with this notion is the observation that the flavonoid abolishes toxicity induced by $ONOO^-$ (Fig. 2B). In addition, mitochondria isolated from quercetin pre-loaded cells were protected against the lipoperoxidative effects of $ONOO^-/CaCl_2$ (Fig. 4A).

Mitochondrial quercetin accumulation bears a second important consequence. The flavonoid may be stored by these organelles and eventually be released, when needed, to promote effects in other subcellular compartments. Several lines of evidence converge in this direction. Loss of detectable quercetin was indeed observed under conditions of exposure to a toxic concentration of $ONOO^-$ (Fig. 2B and inset). An identical outcome was provided by studies, using fluorescence detection at the single cell level, showing time-dependent loss of quercetin in both the cytosolic and mitochondrial compartments (Fig. 5A–E). Hence, prevention of toxicity was associated with oxidation of all the available quercetin, including the fraction stored in the mitochondria. Mitochondrial quercetin, however, may still be oxidized directly, or indirectly, by $ONOO^-$ within mitochondria, thereby making these observations consistent with the notion but not a definitive proof of concept pointing to mitochondria as a reservoir of biologically active quercetin. A critical indication in this direction is however provided by experiments measuring FIC reduction in parallel with quercetin oxidation.

As stated above, FIC is an extracellular oxidant actively reduced by a transplasma membrane oxidoreductase using reducing equivalents provided by electron donors as ascorbic acid [34–38] or quercetin as well as other flavonoids [12,15,16,33]. Details on the specificity of the effects of quercetin are reported elsewhere [12,15,16,33] as well as in the Results section. In this study, extensive FOC formation (Fig. 2A) was found to occur in parallel with quantitative quercetin depletion (inset); interesting results were also obtained measuring quercetin fluorescence at increasing time intervals after FIC addition. Mitochondrial and cytosolic quercetin fluorescence disappeared within 15 min (Fig. 5F), and within the same time point, FOC formation reached a plateau (Fig. 5G). Hence, it appears that in the presence of extracellular oxidants, plasma membrane oxidoreductase activity progressively oxidizes membrane-bound and cytosolic quercetin, continuously replenished via release of the mitochondrial pool of the flavonoid.

The following considerations are necessary to address the physiological relevance of these findings obtained in cultured cells exposed to quite high concentrations of quercetin, a condition which does not allow the formation of quercetin metabolites detected in humans or in experimental animals [46].

Although quercetin aglycone plasma levels are normally low, due to its extensive metabolism [46], circulating glucuronides, sulfates and O-methylated forms of quercetin may also produce beneficial effects [47–49]. In addition, both the flavonoid and the O-methylated flavonoid glucuronides may be de-conjugated by β-glucuronidases present in human tissues, in particular at the inflammatory sites [46,50]. Hence, quercetin (and/or its active metabolites) may indeed reach effective concentrations in tissues. In this perspective, it is important to keep in mind that all the events described in this study (e.g., cellular and mitochondrial accumulation, etc.) were a direct function of quercetin concentration, thereby implying that the same events/effects occur at concentrations below those employed in this study. These considerations allow us to consider very likely the biological relevance of our findings.

In conclusion, the results reported in this study lead to the identification of mitochondria as an important site in which quercetin can be stored to prevent intramitochondrial damage initiated by reactive species. In addition, and/or in alternative, quercetin may

be released when the cytosolic/membrane-bound fraction of the flavonoid is oxidized, thereby implying that mitochondria represent a reservoir of biologically active quercetin.

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