# FLUORESCENCE PROPERTIES OF QUERCETIN IN HUMAN LEUKEMIA JURKAT T-CELLS

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Abstract. We describe the fluorescent properties of the natural flavonoid quercetin in human leukemia Jurkat T-cells. We obtained by spectrofluorimetric measurements that the emission/ excitation spectra of intracellular quercetin displayed a dominant emission maximum at ~540 nm and two different excitation maxima, at ~380 nm and ~440 nm, respectively. Our data suggest that quercetin elicits a specific fluorescence upon binding to intracellular proteins and that there are two dominant molecular configurations of these quercetin-bound proteins which are sensitive to the intracellular $Ca^{2+}$ concentration. Quercetin fluorescence increased substantially after permeabilization of the cells with digitonin, suggesting that intracellular binding of quercetin may be downregulated by a cytosolic factor which is lost by permeabilization. In addition, we performed spectrofluorimetric measurements of the intracellular $Ca^{2+}$ concentration which revealed a strong biphasic calcium release signal after stimulation of intact cells with 50 μM quercetin. The cytosolic $Ca^{2+}$ level increased rapidly from 196 nM to a maximum of 1.7 μM and then declined bi-exponentially to a level of 426 nM, with time constants of 0.48 min. and 4.5 min., respectively. Quercetin also decreased considerably the intracellular NAD(P)H level in intact cells. Taken together, these data add more evidence for the involvement of $Ca^{2+}$ and NAD(P)H in the apoptotic pathway triggered by quercetin in Jurkat cells.

*Key words*: quercetin, fluorescence, Jurkat T-cells, calcium release, NAD(P)H, apoptosis.

## 1. INTRODUCTION

Quercetin (QC) is a widely distributed natural flavonoid which is able to inhibit cell proliferation and induce apoptosis in a specific manner in different cancer cell types [1-4]. Quercetin can activate the apoptotic program *via* the $Ca^{2+}$-dependent mitochondrial pathway, by promoting elevation of cytosolic $Ca^{2+}$ levels, mitochondrial pore opening, collapse of the mitochondrial membrane potential and release of cytochrome *c* from mitochondria [2-5].

However, the data regarding the mechanisms of action of this flavonoid in human leukemia Jurkat T-lymphoblasts remain extremely limited at the moment. It

has been found that quercetin binds to yet unidentified intracellular proteins and thereby accumulates in large quantities in the cytosol and mitochondria of Jurkat cells [6]. In a previous study [1] we have determined that quercetin robustly induces apoptosis in this cell system in a dose- and time-dependent manner. Here we address the question whether QC can promote calcium release in Jurkat cells and assess its effect on the level of the respiratory chain substrate, reduced nicotinamide adenine dinucleotide NAD(P)H. Our results are consistent with the idea that cytosolic $Ca^{2+}$ overload and NAD(P)H hyperoxidation are involved in the induction of apoptosis by quercetin.

In addition, we describe the fluorescence properties of intracellular quercetin in the Jurkat cell system. It is generally known that quercetin is poorly fluorescent in aqueous solutions, but exhibits an increased fluorescence when bound to specific proteins [7,8]. However, to the best of our knowledge, there are no reports on the fluorescence/emission spectra of quercetin in intact cells. The emission spectrum of quercetin in HepG2 cell lysates upon excitation at 488 nm has been reported to present a maximum around 540 nm whose intensity was proportional to the concentration of quercetin [7]. A microscopic visualization of the intracellular fluorescent signal emitted by quercetin at 500-540 nm has been performed in the HepG2 and Jurkat systems with excitation at 488 nm [6,7]. These determinations were obtained in settings compatible with those applied in confocal microscopy. We collected the fluorescence emission/excitation spectra of Jurkat cell suspensions before and after addition of quercetin and from the difference spectra we could derive the specific fluorescence parameters of intracellular quercetin. Monitoring the fluorescence of quercetin could be widely used as a valuable tool in the assessment of its intracellular level, in identifying its target proteins and eventually in a better characterization of its molecular functions.

## 2. MATERIALS AND METHODS

***Cell cultures.*** Human leukemia Jurkat T-cell lymphoblasts were cultured in MegaCell RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified incubator with a 5% $CO_2$ atmosphere. Dihydrated quercetin (Sigma) and fura-2/AM (Invitrogen) were dissolved in dimethyl sulfoxide (DMSO) and kept at -20ºC. Unless stated otherwise, reagents were purchased from Sigma. Cell density, viability and morphology were examined under a phase contrast microscope. Viability was assessed by the trypan-blue exclusion test. Cell count was performed with the use of a haemocytometer.

***Assessment of intracellular*** $[Ca^{2+}]_i$ was performed according to the method of Grynkiewicz et al. [9]. Jurkat cells were washed twice in a standard saline solution (SS) containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10

mM HEPES, 10 mM glucose, pH 7.2/NaOH. The cells were then incubated with 2.5 μM fura-2/AM for 20 min. in the dark at room temperature (24ºC) with occasional agitation, then washed twice in SS, resuspended in SS at a density of ~$10^6$ cells/ml, incubated at 37ºC for an additional 40 min. and then transferred into a 2 ml quartz cuvette maintained at 37ºC under continuous stirring. Cell viability assessed immediately before the fluorescence recordings was at least 90% in all cell samples. Fluorescence was recorded with a Horiba Jobin Yvon spectrofluorimeter, by sequential excitation at 340 and 380 nm. The excitation pulses were repeated every 20-30 s. Emission was collected at 490 nm to reduce the interference with QC fluorescence. Quercetin was added after the fluorescence signal became stable following an initial pre-equilibration period (30 min. of recording). The intracellular $Ca^{2+}$ concentration, $[Ca^{2+}]_i$, was calculated from the equation:

$$\left[\mathrm{Ca}^{2+}\right]_i = \beta K_D \left(R - R_{\min}\right) / \left(R_{\max} - R\right)$$

where $R = F_{340}/F_{380}$ represents the ratio of the fluorescence emission at 340 nm and 380 nm excitation, respectively, $R_{\min}$ is the ratio $F_{340}/F_{380}$ obtained in a nominally $Ca^{2+}$-free solution, $R_{\max}$ is $F_{340}/F_{380}$ obtained when the $Ca^{2+}$ indicator is saturated with $Ca^{2+}$, $\beta$ is the ratio between $F_{380}$ in the $Ca^{2+}$-free solution and $F_{380}$ at saturation, and $K_d$ = 0.225 μM is the $Ca^{2+}$-dissociation constant of the indicator. Calibration for $R_{\max}$ was performed at the end of each recording by addition for 15 min. of 35 μM digitonin from a stock solution of 100 mM in DMSO. The degree of cell permeabilization was 100% as evaluated by trypan blue tests in separate determinations. Finally, $R_{\min}$ was evaluated after addition of 20 mM EGTA from a stock solution of 0.75 M, pH 7.2, which lowered the free $Ca^{2+}$ concentration to 7 nM as calculated with the WEBMAXCLITE v1.15 software (http://maxchelator.stanford.edu). Control cells were treated in the same manner with the exception that an equal amount of DMSO was added instead of the calcium indicator. All values were corrected for autofluorescence.

***Fluorescence spectroscopy.*** The cell suspensions were prepared at a density of ~$10^6$ cells/ml in SS after three washes with SS and transferred to a 2 ml quartz cuvette under continuous stirring. Fluorescence was recorded at 37ºC with a Horiba Jobin Yvon spectrofluorimeter. The first spectrum was recorded after a pre-equilibration period of 35 min. Excitation/emission spectra were collected before and after quercetin addition to the cuvette at indicated times. After 1 hour of exposure to QC, 35 μM digitonin was added to the cuvette. Fluorescence spectra were collected after 15 min., then 10 mM EGTA was added and a new series of spectra were recorded after an additional 15 min. In this final step, the calculated $Ca^{2+}$ concentration was 15 nM. Control cells were exposed to DMSO instead of quercetin.

All the data presented in this paper were obtained from at least three different experiments.

## 3. RESULTS

By spectrofluorimetric measurements of fura-2/AM loaded cells, we determined that 50 μM QC induced a consistent calcium release from intracellular stores, which generated a distinctive, biphasic $Ca^{2+}$ signal (Fig. 1). The initial rising phase was rapid, and a maximum of 1.67 μM was reached within 1.2 min. after the addition of quercetin to the cell suspension. The subsequent decay phase of the $Ca^{2+}$ signal was best fitted to a double-exponential equation, with the corresponding time constants of 0.484 ± 0.086 min. and 4.54 ± 0.17 min., respectively. The final steady level obtained from the fit was 425.7 ± 2.8 nM, which was consistently higher than the initial resting concentration, 196.4 ± 29.9 nM (the latter value is similar to previous reports [10,11]).

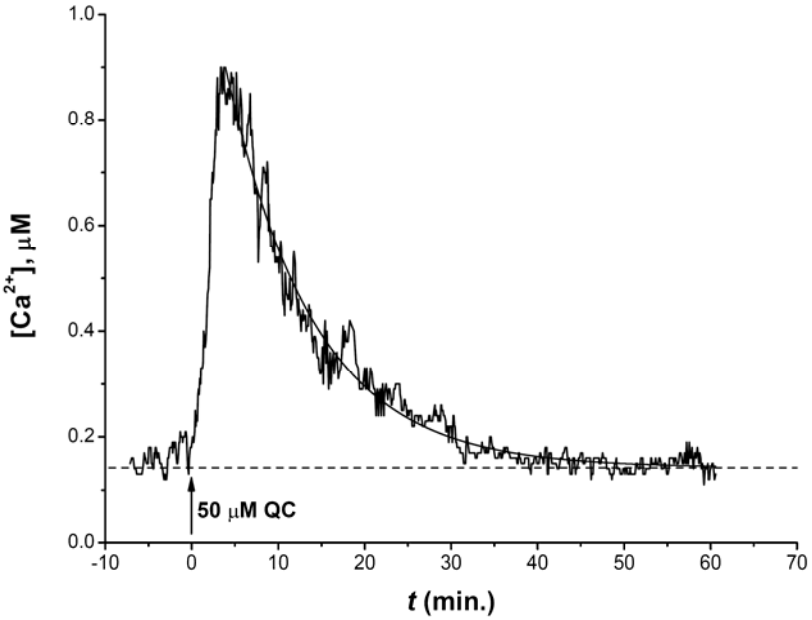


Fig. 1 – 50 μM quercetin induces a transient increase in the level of intracellular $Ca^{2+}$ in Jurkat cells. The calcium signal was recorded by spectrofluorimetry in fura-2/AM loaded cells, and the average trace obtained from 5 different experiments is shown. The decay phase of the signal was fitted to a double exponential equation (solid smooth line) within the Origin software environment. The resting level obtained from average over the initial period is indicated (dashed line). QC was added at $t = 0$.

In order to better characterize the fluorescent properties of intracellular quercetin, we monitored the changes in its fluorescence spectra during exposure of intact Jurkat cells to 50 μM QC and after cell permeabilization with digitonin.

First, we recorded the QC emission spectra after excitation at 460 nm of intact cell suspensions after 15 and 60 min. of incubation with the flavonoid (Fig. 2).

A prominent maximum centered at 546 nm was observed in both cases. In addition, the fluorescence intensity at this wavelength appeared to increase progressively during continuous exposure to QC (not shown). After the addition of digitonin in the cuvette, which elevated the cytosolic $Ca^{2+}$ level to 1 mM, the QC emission spectrum became wider but its maximum remained centered at about 540 nm (Fig. 2). An additional maximum could be distinguished at ~515 nm, which presumably corresponds to the mitochondrial bound quercetin [6]. However, under our conditions this maximum did not contribute significantly to the total emission of quercetin-loaded intact cells (e.g., curves "*a*" and "*b*" in Fig. 2). Next, after lowering the $Ca^{2+}$ concentration to 15 nM by EGTA addition, the QC emission spectrum remained qualitatively similar to that obtained at 1 mM $Ca^{2+}$, but exhibited a significant reduction in intensity (Fig. 2).

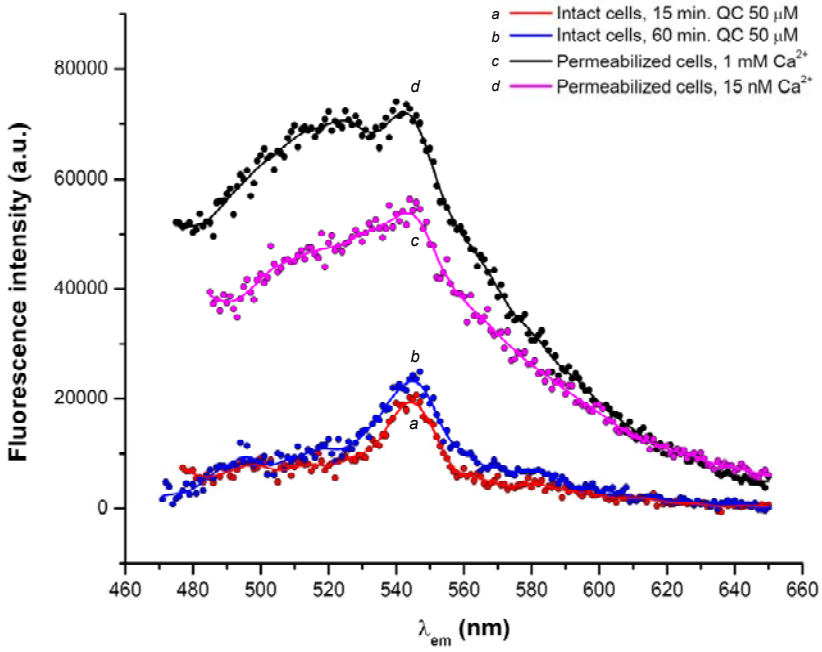


Fig. 2 – Fluorescence emission spectrum of intracellular quercetin upon excitation at 460 nm. Spectrofluorimetric determinations were done after 15 and 60 min. from addition of QC to the Jurkat cell suspension, and then 15 min. after permeabilization with digitonin in a saline solution containing 1 mM $Ca^{2+}$. Finally, EGTA was added to the cuvette to reach a final $Ca^{2+}$ concentration of 15 nM. All values were corrected for autofluorescence. The raw data (circles) were smoothed by a 7-point FFT Origin algorithm (solid lines).

We also determined the excitation spectra of intracellular QC, under the same conditions as described above, with emission at 527 nm and 544 nm, respectively. In Fig. 3 we present typical excitation spectra obtained with emission at 527 nm. In

intact cells, two specific maxima were evident at 380 nm and 440 nm, respectively. The amplitude of both maxima increased continuously during exposure to QC (Fig. 3 and data not shown). Interestingly, after permeabilization in the 1 mM $Ca^{2+}$ solution both maxima displayed a consistent increase above the values observed in intact cells, but then exhibited different responses to the reduction of the $Ca^{2+}$ level to 15 nM. These data indicate that two different molecular states of intracellularly bound quercetin exist which are $Ca^{2+}$-sensitive. The fluorescence of the 380 nm-state decreased, whereas that of the 440 nm-state increased when the $Ca^{2+}$ concentration was reduced from 1 mM to 15 nM (Fig. 3). When emission at 544 nm was recorded under otherwise similar conditions, a similar behavior was observed (not shown).

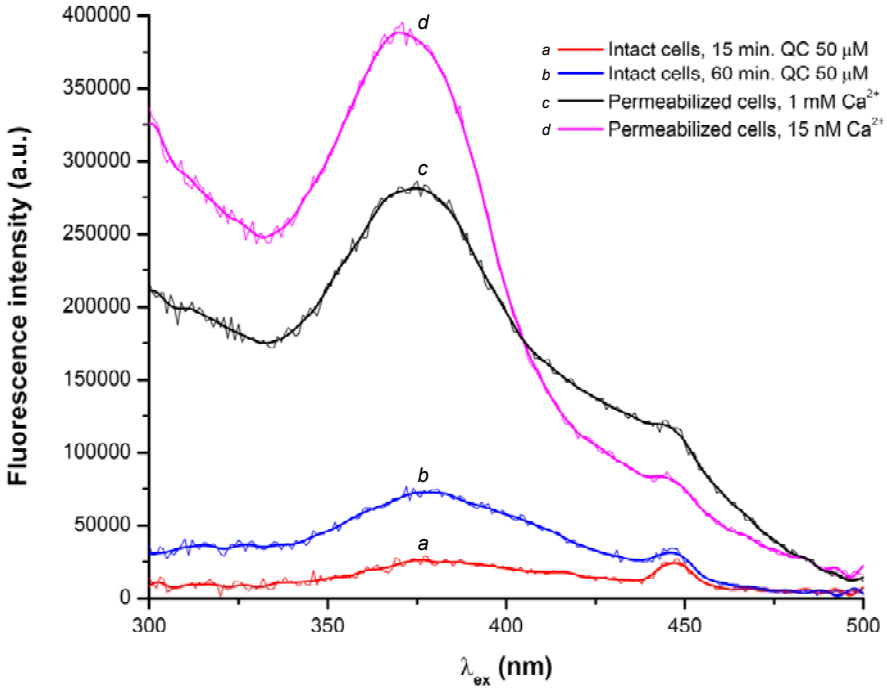


Fig. 3 – Fluorescence excitation spectra of intracellular quercetin, with emission at 527 nm. Other details are as in Fig. 2.

The emission spectrum of control cells obtained with excitation at 440 nm (Fig. 4A) displayed the general characteristic features of NAD(P)H [12,13] with a rather wide spectrum. However, a clear sharp maximum was visible at about 515 nm, which is specific to mitochondrial flavins such as FMN and FAD [12]. Fluorescence emission increased after exposure to quercetin for 15 min., and the difference spectrum displayed a distinct maximum at 536 nm (Fig. 4B).

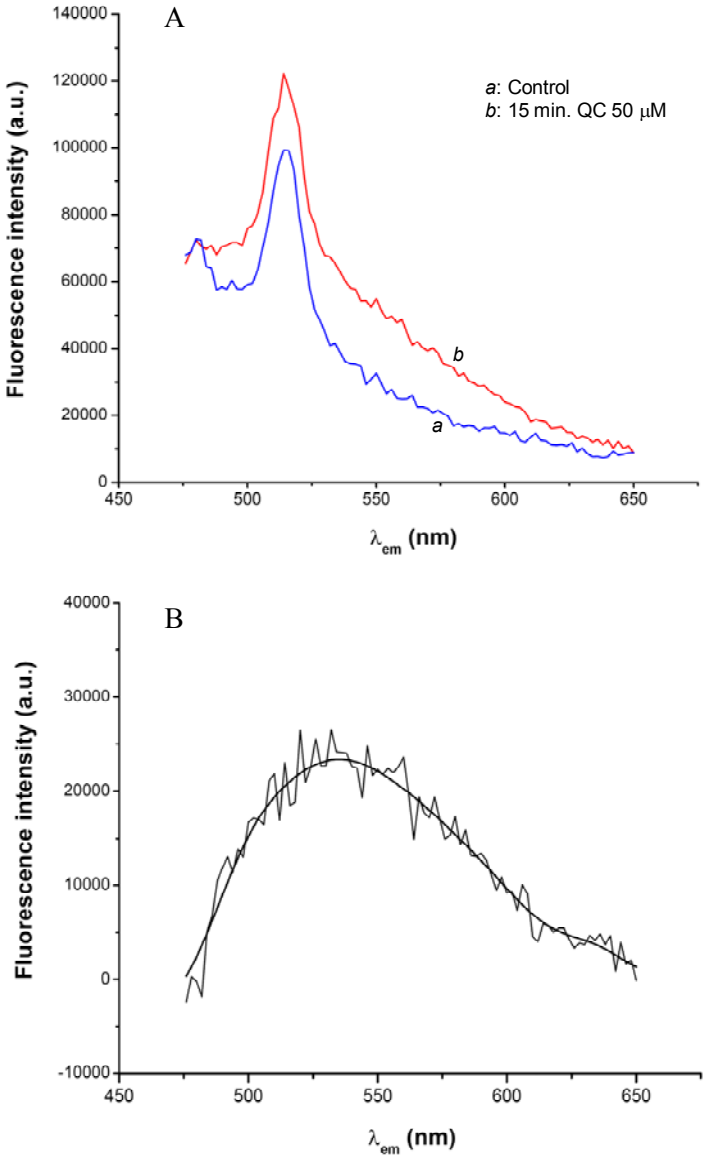


Fig. 4 – Fluorescence emission spectrum of intracellular quercetin, with excitation at 440 nm. (A) Spectrofluorimetric determinations were done before ("*a*") and after 15 min. ("*b*") from addition of 50 μM QC to the cell suspension. (B) The difference spectrum obtained after subtraction (*b* minus *a*). The data were smoothed by an 8-point FFT Origin algorithm (smooth line).

The fluorescence emission spectrum of control cells obtained with excitation at 380 nm also displayed the characteristic features of NAD(P)H [12,13], with a maximum at 435 nm (Fig. 5A). The curve "*a*" in Fig. 5A is closely similar to the

NAD(P)H emission spectrum presented in Fig. 6B of [13]. Fluorescence emission at ~540 nm increased after exposure to quercetin for 15 min., and the difference spectrum displayed a distinct maximum centered on 545 nm (Fig. 4B). However, addition of quercetin appeared to decrease the level of NAD(P)H, which is reflected clearly in the fluorescence reduction in the domain $\lambda < 500$ nm. This reduction can be better observed as a negative deflection in the corresponding region of the difference spectrum presented in Fig. 4B.

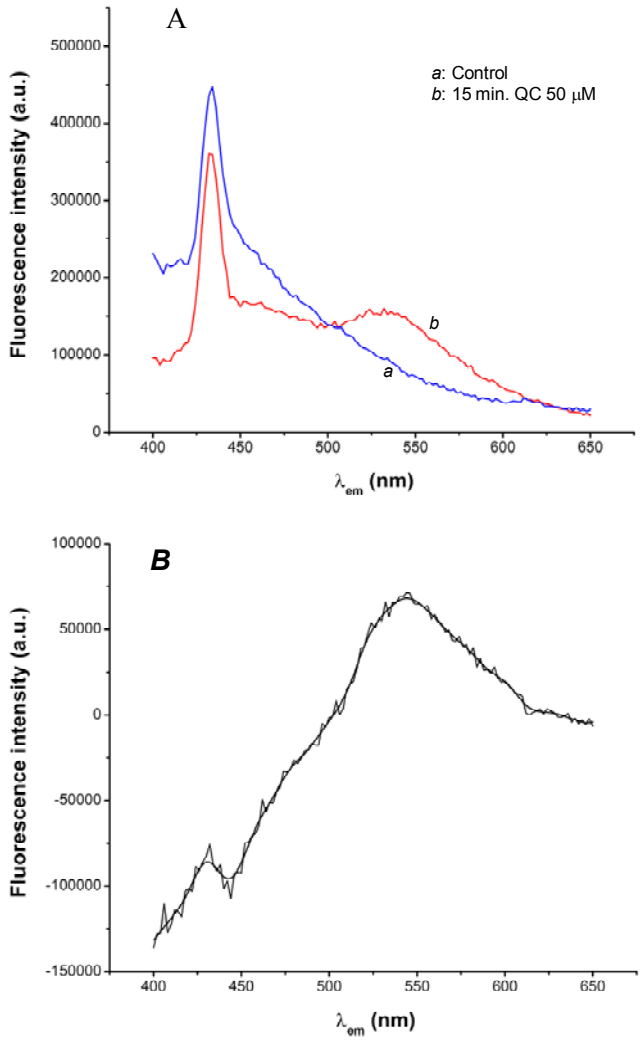


Fig. 5 – Emission spectrum of intracellular quercetin, with excitation at 380 nm. Other details are as in Fig. 4.

The data described here are in good agreement with the fluorescence properties of quercetin reported for aqueous solutions, cell lysates or intact cells [6-8]. In different aqueous phosphate buffered solutions, QC has been found to present a reduced fluorescence with an emission maximum at 535 nm [8] or 500 nm [7] and optimal excitation at 373 nm [8] or 430 nm [7]. Collectively, all these results indicate clearly that the maxima observed in the difference spectra presented here correspond to quercetin bound intracellularly in the Jurkat cell system.

The kinetic variations in the intracellular NAD(P)H levels can be best monitored by recording the fluorescence emitted at ~430-450 nm after excitation at ~340-360 nm [12,13]. The oxidized form (NAD+) of this molecule is non-fluorogenic, therefore a reduction in the fluorescence intensity detected at these wavelengths indicates a decrease in the NAD(P)H level. By kinetic recordings of the fluorescence emitted at 435 nm after excitation at 360 nm, we obtained in five different experiments that 50 μM QC depressed ~3-5 times the level of NAD(P)H within ~2 min., and this reduction persisted for at least 1 h from application (not shown).

## 4. DISCUSSION

This study clearly shows that quercetin accumulates inside Jurkat cells and binds to intracellular proteins, thereby exhibiting a specific fluorescence with emission at ~540 nm. Under our experimental conditions, intracellular quercetin displayed two specific excitation maxima, at ~380 nm and ~440 nm, which may correspond to two different proteins that bind quercetin, or alternatively to two different states of a unique quercetin-bound protein. Moreover, we found that both states are sensitive to the intracellular $Ca^{2+}$ level and display different effects upon the reduction of $[Ca^{2+}]_i$ from very high (1 mM) to very low (15 nM) values.

In addition, a secondary emission maximum became visible at ~515 nm in digitonin-permeabilized cells upon excitation at 460 nm, which most likely originates from mitochondrial-bound quercetin [6]. This maximum could not be clearly distinguished after excitation at 440 nm, when the apparent emission maximum shifted from 540 nm to 510 nm (not shown), indicating the involvement of a second molecular state with emission at 490 nm as estimated by a Gaussian deconvolution analysis. Thus, emission in the 490-520 nm window with excitation at 460-490 nm might be a better indicative for the mitochondrial-bound quercetin, whereas emission at 530-560 nm with excitation at 380 nm and 440 nm probably reflects the binding of the flavonoid to a non-diffusible cytosolic protein or to a protein found in a specific intracellular organelle, different from the mitochondrion. We are currently investigating the dependence of the fluorescent properties of these molecular states on the intracellular $Ca^{2+}$ concentration.

Quercetin fluorescence increased substantially after permeabilization of the cells with digitonin, which was quite surprising since we expected that the perfusion with the extracellular solution should reduce the availability of cytosolic

QC-binding proteins or promote the efflux of quercetin from intracellular compartments. Hence, our results suggest that intracellular binding of quercetin may be downregulated by a cytosolic factor which is lost by permeabilization.

We also assessed that 50 μM quercetin induced a strong $Ca^{2+}$ release signal in Jurkat cells, which elevated rapidly the cytosolic $Ca^{2+}$ level from 196 nM to 1.7 μM. The subsequent decline of $[Ca^{2+}]_i$ evolved slowly, on a time scale of the order of ~10 min., and a steady level of 426 nM was eventually reached, which was consistently higher than the resting $[Ca^{2+}]_i$. These findings suggest that quercetin promotes $Ca^{2+}$ mobilization from intracellular stores, possibly by acting as an activator of a $Ca^{2+}$ release channel [14,15]. At the same concentration, quercetin also decreased rapidly the intracellular NAD(P)H level, which remained low for at least 1 h after stimulation. Possible causes for this decrease may be the hyperoxidation of NAD(P)H following exposure to quercetin or the degradation of the molecule by direct or indirect action of free radicals [16]. However, we have found that under these particular conditions of exposure, quercetin exhibits a protective effect against $H_2O_2$ [1]. In addition, with the use of a fluorescent probe for the superoxide anion we did not detect any increase in the mitochondrial superoxide level during the exposure to 50 μM quercetin for 1 h (not shown). Consequently, our data strongly suggest that QC promotes NAD(P)H hyperoxidation, which may be further amplified via $Ca^{2+}$-induced mitochondrial depolarization [17]. Moreover, the cytosolic $Ca^{2+}$ overload and NAD(P)H hyperoxidation can act synergistically to trigger mitochondrial pore opening [16]. We also determined by flow cytometer measurements that incubation of Jurkat cells with 50 μM quercetin for 1 h induces significant apoptosis (not shown). Taken together, these data, corroborated by the overall strong ability of quercetin to induce apoptosis in Jurkat cells [1], present clear evidence for the involvement of $Ca^{2+}$ and NAD(P)H in the apoptotic program triggered by quercetin in Jurkat cells.

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