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Resveratrol is not compatible with a Fura-2-based assay for measuring intracellular Ca^{2+} signaling

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ABSTRACT

Fura-2 is a commonly used fluorescent Ca^{2+} dye that allows an accurate determination of cytosolic Ca^{2+} levels by measuring the emission obtained at 510 nm after alternating excitation at 340 nm and 380 nm (F_{340}/F_{380} ratio). Previous studies, based on Fura-2 measurements, claimed that resveratrol, a polyphenol implicated in human health, triggered an acute rise in cytosolic [Ca^{2+}]. In this report, we show that the spectral properties of resveratrol are not compatible with the fluorescent properties of Fura-2. Resveratrol displays a strong absorption of light at a wavelength of 340 nm and a strong emission at 510 nm upon excitation at 340 nm (F_{340}). As a consequence, the F_{340} values, but not the F_{380} values, are increased when incubating cells with resveratrol. Consequently the F_{340}/F_{380} ratio values acutely increase upon addition of resveratrol, independently of changes in cytosolic [Ca^{2+}]. Yet, we show that pretreating cells with resveratrol does not affect the F_{340}/F_{380} ratios of Fura-2, provided that resveratrol is washed away before fluorescence measurement. These results indicate that Fura-2 is not suitable for assessing acute effects of resveratrol on Ca^{2+} signaling but that long-time effects can be assessed, provided that the resveratrol is carefully removed by appropriate wash steps.

1. Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a polyphenol produced against environmental stress by many plant species [1,2]. The intake of this polyphenol has been implicated to be beneficial for human health, including longevity and anti-ageing properties [3] and protection against diseases like neurodegenerative disorders [4], metabolic disorders [5] and different types of cancers [6]. Resveratrol may activate different energy-sensing regulators like AMPK and sirtuin 1 by inhibiting cAMP phosphodiesterases [3].

Resveratrol has also been proposed to act as an agent that mobilizes intracellular Ca^{2+} [3,7–10]. In different studies, the acute effects of resveratrol on intracellular Ca^{2+} homeostasis and dynamics were assessed using the fluorescent ratiometric Ca^{2+} dye, Fura-2-AM [8–13]. Upon Ca^{2+} binding, Fura-2 displays a shift in its excitation profile from 380 nm to 340 nm [14,15]. As such, measuring the emission of Fura-2 at 510 nm after alternating excitation with 340 nm and 380 nm light and plotting the F_{340}/F_{380} ratio has been recommended for the measurement of intracellular Ca^{2+} signals [15]. Although resveratrol has been shown to absorb light in the UV spectrum [16], it has been used in combination with Fura-2 to study its effect on Ca^{2+} signaling, leading to the conclusion that resveratrol can trigger intracellular Ca^{2+} release [8,9].

Hence, we wanted to scrutinize the compatibility of resveratrol with the use of Fura-2 for measuring the effects of resveratrol on intracellular Ca^{2+} dynamics. Here, we show that using HeLa cells as an experimental cell model, resveratrol interferes with the Fura-2 emission upon excitation at 340 nm, thereby directly affecting the F_{340}/F_{380} ratio independently of any changes in [Ca^{2+}]. As such, we propose that Fura-2 should not be used as a ratiometric dye to study acute effects of resveratrol on intracellular Ca^{2+}-signaling events and advice caution when using compounds absorbing in the UV-part of the spectrum in Fura-2-based Ca^{2+} assays.

2. Materials and methods

2.1. Materials

Fura-2-AM was from Invitrogen; resveratrol (*trans*-3,4′,5-trihydroxystilbene) was from Calbiochem; dimethyl sulfoxide (DMSO) was from Sigma; and thapsigargin was from Alomone Labs. The stock solutions of resveratrol (200 mM in DMSO), of Fura-2-AM (1.25 mM in DMSO) and of thapsigargin (2 mM in

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ethanol) were stored at −20 °C for long-term use. Resveratrol and Fura-2-AM were always shielded from direct light.

2.2. Cell culture

Wild-type human cervix carcinoma cells (HeLa cells) were cultured at 37 °C and 5% CO_2 in GlutaMAX-containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 10 mM HEPES buffer. The cells were cultured in the presence of 85 IU ml^{-1} penicillin and 85 μg ml^{-1} streptomycin [17].

2.3. Fura-2 measurements in intact HeLa cells in the presence or absence of resveratrol

HeLa cells were seeded in a 96-well plate (1×10^4 per well). Two days later, cells were treated or not with resveratrol at the concentrations indicated in the figures for 4 h. Subsequently, the cell culture medium was removed and the cells were washed once with modified Krebs solution (135 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.6 mM HEPES, 11.5 mM glucose and 1.5 mM Ca^{2+}). Cells were subsequently loaded with the ratiometric dye Fura-2-AM (1.25 μM) for 30 min at room temperature in modified Krebs solution. Cells were then washed with modified Krebs solution and incubated for 30 min at room temperature. Depending on the aim of the experiment, resveratrol was either present or absent during the two latter steps, as will be further explained. Fluorescence was subsequently measured on a Flexstation® 3 benchtop multi-mode microplate reader (Molecular Devices) by alternatively exciting the dye at 340 nm and 380 nm while measuring the fluorescence emission at 510 nm [17]. When indicated, EGTA (final concentration of 3 mM) and thapsigargin (final concentration of 1 μM) were added to the cells to respectively chelate extracellular Ca^{2+} and to evoke Ca^{2+} release from the endoplasmic reticulum.

2.4. Measurement of a direct effect of resveratrol on Ca^{2+} signaling in intact cells

To measure the acute effect of resveratrol on Ca^{2+} signaling, we cultured the cells as mentioned above in the absence of resveratrol. No resveratrol was added to the cells during incubation with Fura-2-AM and the 30-min wash step. A final concentration of 3 mM of EGTA was added to the cells for extracellular Ca^{2+} chelation and Fura-2 ratio measurement was initiated. Resveratrol (100 μM) or DMSO (as control) was subsequently added automatically to the cells 1 min later.

3. Results

3.1. Resveratrol directly interferes with F_{340}/F_{380} ratio values of Fura-2

To assess the effect of resveratrol on Fura-2-based Ca^{2+} assays, we first investigated its effect in cells, which were pre-treated with resveratrol (100 μM) for 4 h as well as during the Fura-2 loading step and the wash step. By comparing the resveratrol-treated cells with the vehicle (DMSO)-treated cells, we found that the emission values obtained in resveratrol-treated cells at 510 nm upon excitation at 340 nm (F_{340}) were consistently higher than the ones obtained in vehicle-treated cells (Fig. 1A). This was observed before and after thapsigargin addition. Importantly, F_{380} values were very comparable between both conditions, suggesting that the effects of resveratrol on the F_{340} values were not related to changes in intracellular [Ca^{2+}] (Fig. 1A). As a consequence, resveratrol-treated cells displayed higher F_{340}/F_{380} values than vehicle-treated cells did (Fig. 1B). Subsequently, in the next set of experiments, we assessed the acute effect of resveratrol on the F_{340}/F_{380} ratio in EGTA-exposed Fura-2-loaded HeLa cells, as is classically performed when assessing the ability of compounds to mobilize Ca^{2+} from intracellular Ca^{2+} stores. Hence, the vehicle or resveratrol was added by an automated pipetting system upon continuously monitoring the F_{340}/F_{380} ratio of Fura-2-loaded HeLa cells. While the vehicle alone (DMSO) neither affected the F_{340} values nor the F_{380} values (Fig. 2A), resveratrol (100 μM) induced an immediate increase in the F_{340} values while there was no such change in F_{380} values. As a consequence, resveratrol (100 μM), but not the DMSO vehicle control, caused a simultaneous increase in the F_{340}/F_{380} ratio of Fura-2 (Fig. 2C).

3.2. Resveratrol absorbs light in the range of 340 nm wavelength and emits light at 510 nm upon excitation at 340 nm

The interference of resveratrol on Fura-2 signaling prompted us to carefully analyze the absorption spectrum of resveratrol (Fig. 3A). We found that resveratrol displayed a broad absorption peak between 290 nm and 360 nm with a maximum around 320 nm, which could be observed for resveratrol concentrations of 10 μM or higher (Fig. 3A). As a consequence, resveratrol displayed in a concentration-dependent manner a clear absorption at 340 nm, but not at 380 nm, correlating with the fact that resveratrol affects F_{340} but not F_{380} values of Fura-2. Most importantly, by examining the spectral properties of different resveratrol concentrations in the absence of cells and of Fura-2, we found that resveratrol emitted light at 510 nm while excited at 340 nm (Fig. 3B). As a result, a clear upward shift in the F_{340}/F_{380} values was

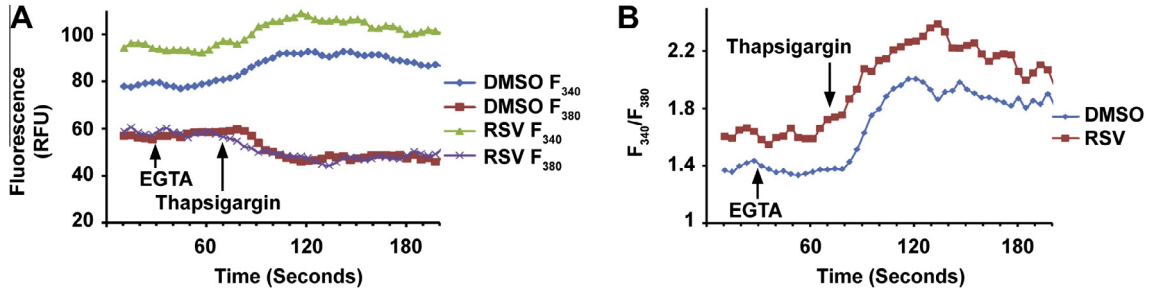

Fig. 1. Resveratrol-pretreated HeLa cells display a higher basal F_{340} value and a higher F_{340}/F_{380} ratio. HeLa cells were seeded in a 96-well plate (10,000 cells/well) and treated after two days with the vehicle (DMSO) or 100 μM resveratrol (RSV) for 4 h. Resveratrol and DMSO were also added during the Fura-2-AM incubation step and the 30-min wash step. EGTA (3 mM) was added as indicated to chelate all extracellular Ca^{2+}, 30 s after the start of the fluorescence measurement. To uncover the endoplasmic reticulum Ca^{2+} leak, thapsigargin (1 μM) was added as indicated. (A) Fluorescence is represented in relative fluorescence units (RFU). Representative traces are shown ($n = 3$). (B) Representative traces depicting the F_{340}/F_{380} ratios ($n = 3$).

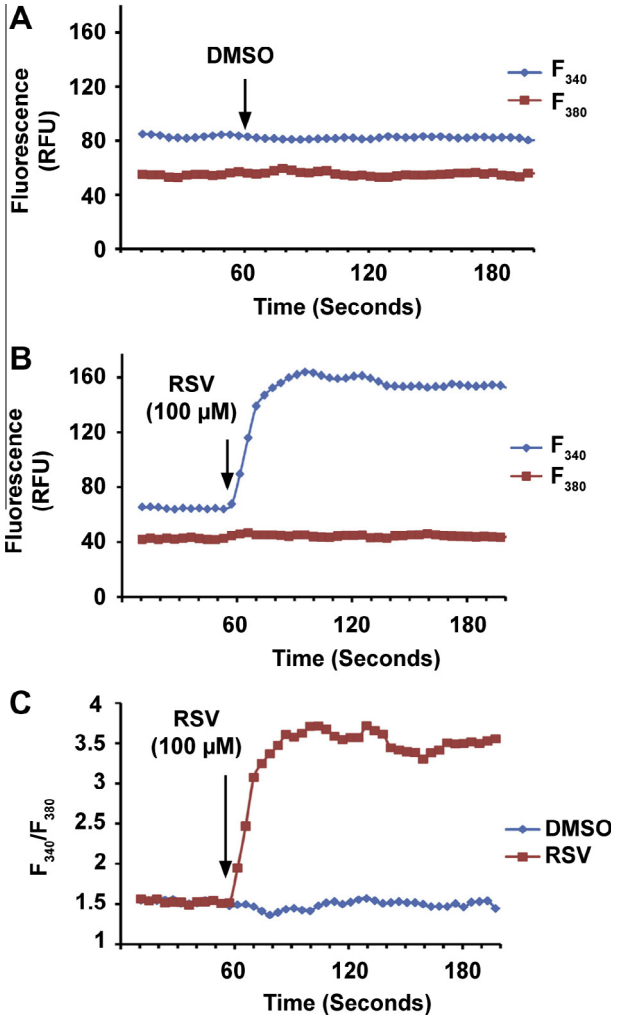

Fig. 2. Acute addition of resveratrol interferes with the F_{340} value and the F_{340}/F_{380} ratio of Fura-2. HeLa cells were seeded in a 96-well plate (10,000 cells/well) and were grown for two days in the absence of resveratrol. The cells were incubated with Fura-2-AM for 30 min followed by a 30-min wash step. EGTA (3 mM) was added to chelate extracellular Ca^{2+} just before the start of the fluorescence measurement. Subsequently, (A) DMSO as a vehicle or (B) resveratrol (100 μM) was added as indicated and fluorescence was measured and expressed in relative fluorescence units (RFU). Representative traces are shown ($n = 3$); (C) representative traces of the F_{340}/F_{380} ratio obtained in (A) and (B) ($n = 3$).

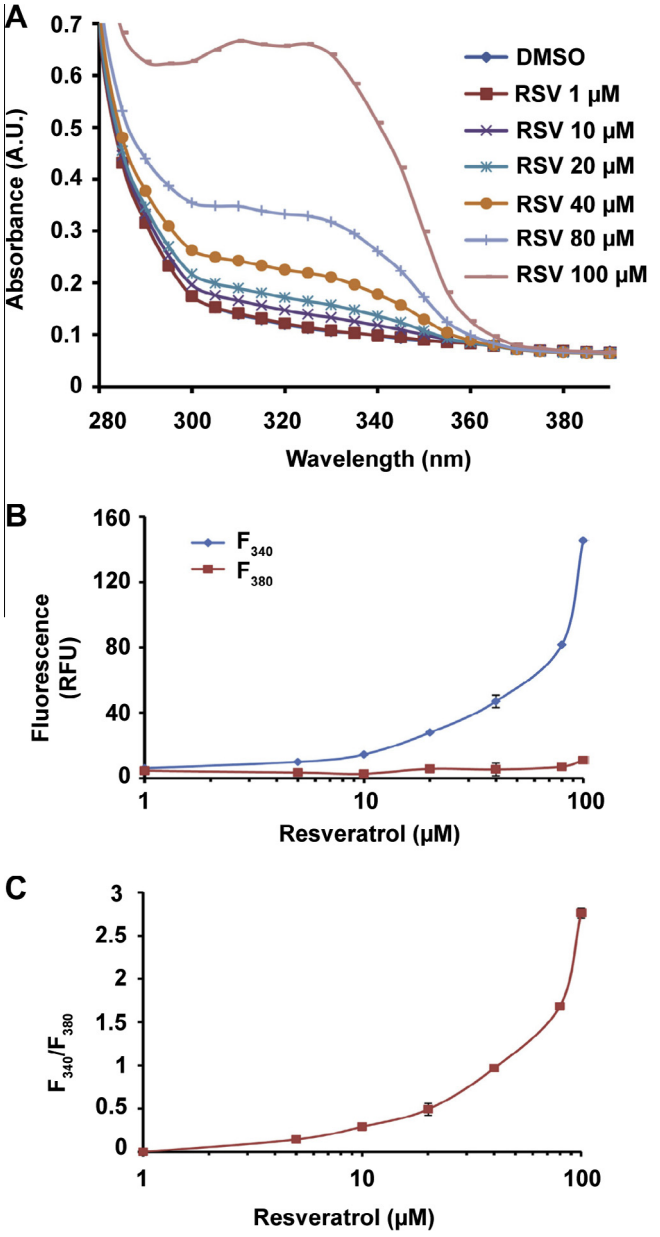

Fig. 3. Resveratrol absorbs light in the UV part of the spectrum and emits at 510 nm when excited at 340 nm. (A) The absorption spectrum of resveratrol (RSV) was measured at various wavelengths using the Flexstation® 3. Absorbance is given in absorbance units (A.U.). Different concentrations of resveratrol (1–100 μM) were used as indicated. The absorption spectrum of 1 μM resveratrol overlaps with that obtained for the vehicle (DMSO). Representative traces are shown ($n = 3$). (B) Emission at 510 nm obtained by the indicated concentrations of resveratrol in the absence of cells and of Fura-2. Fluorescence measured (in relative fluorescence units, RFU) for the vehicle control condition (DMSO) was subtracted from the fluorescence values obtained for the different concentrations of resveratrol. Excitation was at alternating 340 nm and 380 nm wavelengths. The representative data are presented as F_{340} and F_{380} ($n = 3$). (C) Representative traces of the F_{340}/F_{380} obtained in (B) ($n = 3$).

observed, starting at 5 μM, but becoming very prominent at increasing concentrations (Fig. 3C).

3.3. Proper wash steps eliminate the interference of resveratrol with Fura-2 based Ca^{2+} measurements

Since resveratrol is cell permeable, we examined whether pre-treatment of the cells with resveratrol (100 μM) but omitting it during Fura-2 incubation and the subsequent washing step would affect the F_{340} values and thus the F_{340}/F_{380} values of Fura-2. Importantly, we found that 1 h in resveratrol-free conditions was effective to eliminate the interference of up to 100 μM resveratrol on the fluorescent properties of Fura-2, since F_{340} values (Fig. 4A) and thus F_{340}/F_{380} values (Fig. 4B) were not different between DMSO-pretreated and resveratrol-pretreated cells. This indicates that Fura-2 measurements are in principle compatible with resveratrol pre-treatment of cells, provided adequate wash steps are followed.

4. Discussion

Resveratrol can act in the cell on different mechanisms like activation of AMPK and sirtuin 1 [3,7] and therefore may affect indirectly the Ca^{2+}-handling machinery of the cell. However, in view of its pleiotropic effects, resveratrol may equally well affect in a direct way cellular Ca^{2+}-signaling mechanisms. Interestingly, different studies have implicated that resveratrol affects intracellular

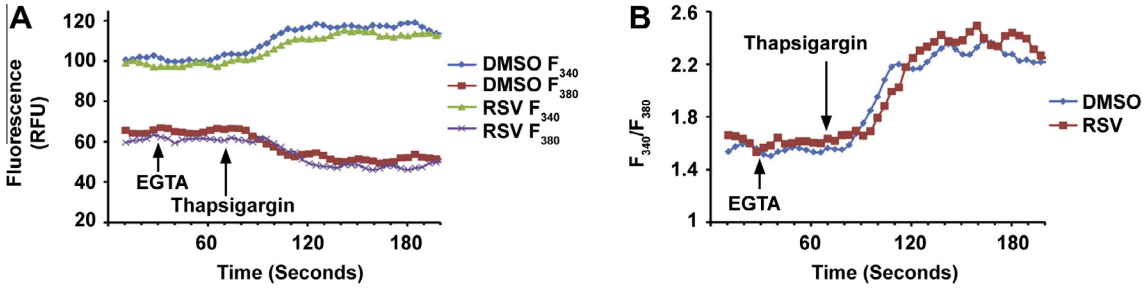

Fig. 4. The interference of resveratrol with the Fura-2 signal can be abolished by a prior wash step. HeLa cells were seeded in a 96-well plate (10,000 cells/well) and treated after two days with the vehicle (DMSO) or 100 μM resveratrol (RSV) for 4 h. Resveratrol and DMSO were absent during the Fura-2-AM incubation step and the 30-min wash step. EGTA (3 mM) was added as indicated to chelate all extracellular Ca^{2+}, 30 s after the start of the fluorescence measurement. To uncover the endoplasmic reticulum Ca^{2+} leak, thapsigargin (1 μM) was added as indicated. (A) Fluorescence is represented in relative fluorescence units (RFU). Representative traces are shown ($n = 3$). (B) Representative traces depicting the F_{340}/F_{380} ratios ($n = 3$).

Ca^{2+} homeostasis and dynamics [8–11]. Yet, many of these Ca^{2+} measurements were based on the commonly used ratiometric fluorescent Ca^{2+} dye, Fura-2. Hence, the main purpose of this study was to examine whether Fura-2-based Ca^{2+} measurements by using F_{340}/F_{380} ratios can be used to detect resveratrol-mediated effects on Ca^{2+} signaling in intact cells.

In the present study, we firstly showed that resveratrol can interfere with the F_{340}/F_{380} ratios of intracellular Fura-2, which is commonly used as a measure of intracellular [Ca^{2+}]. Secondly, the absorption of light by resveratrol at 340 nm, as had previously been shown [16,18], overlaps with the absorption spectrum of Fura-2. Consequently, resveratrol clearly interfered with the F_{340} value of Fura-2, as resveratrol emits at 510 nm after excitation at 340 nm. This occurs in the presence as well as in the absence of Fura-2, which fits with previous studies on the spectral properties of resveratrol [18–20]. These results demonstrate that resveratrol, at concentrations of ~10 μM and higher, strongly interferes with the fluorescent properties of Fura-2 used to assess intracellular Ca^{2+} signaling. In view of this result, we conclude that the ratiometric dye Fura-2-based Ca^{2+} measurements are not compatible for studying the acute effect of resveratrol on intracellular Ca^{2+} dynamics, in particular when using resveratrol concentrations of 10 μM and higher.

Importantly, our data also demonstrated that to study long-term effects of resveratrol on Ca^{2+} signaling, simple wash steps are sufficient to avoid the interference of resveratrol on Fura-2 fluorescence measurement. Finally, our data are in full agreement with the very recent work by Kopp et al. [21], who showed that resveratrol interfered with Fura-2-based Ca^{2+} imaging experiments but not with Fluo-4 or YC3.60 Ca^{2+} indicators in MDA-MB-231 cells.

These data underscore the importance of choosing the right fluorescent Ca^{2+} dyes and of carefully inspecting the spectral properties of chemical compounds in order to make justified claims about the ability of chemical compounds, like resveratrol, to trigger intracellular [Ca^{2+}] changes. In particular, our data together with the very recent report of Roe and coworkers [21] convincingly argue against the use of Fura-2-based assays to assess the acute effects of resveratrol on intracellular Ca^{2+} signaling or to explain the biological or cellular effects of resveratrol through changes in intracellular Ca^{2+} homeostasis and dynamics, in contrast with what was previously published [8,9]. As such, the effects of resveratrol on an acute modulation of the intracellular Ca^{2+} signaling or acute rise of cytosolic [Ca^{2+}] should be revisited using fluorescent Ca^{2+} dyes or genetically encoded Ca^{2+} sensors displaying fluorescent properties compatible with resveratrol. Importantly, our study also indicates that proper wash steps can be applied to study the effect of prolonged resveratrol treatment of cells on intracellular Ca^{2+} homeostasis and dynamics.

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