Changes of intracellular Ca\(^{2+}\) in quercetin-induced autophagy progression

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Abstract

Quercetin was previously reported to exhibit significant anti-proliferative activities, and its major effect on tumors was to induce cell apoptosis or autophagy. However, the specific mechanism remains controversial. In this study, autophagy induced by quercetin was determined with various methods. Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{i}}\)) was measured after being incubated with Fluo-3 acetoxymethyl (AM). At the same time, the relationship between the intracellular Ca\(^{2+}\) and autophagy induced by quercetin was further analyzed. These results showed that autophagy induced by quercetin (0–50 µg/ml) in HepG2 cells was in a dose-dependent manner. Meanwhile, when autophagy was induced by quercetin, [Ca\(^{2+}\)]\(_{\text{i}}\) was significantly increased. And after being incubated with calcium chelator 1,2-bis(2-aminophenoxy)ethane-N\(_2\),N\(_2\),N\(_2\),N\(_2\)’-tetraacetic acid-AM, autophagy was suppressed, which implied that [Ca\(^{2+}\)]\(_{\text{i}}\) elevation appeared to be the cause for autophagy induction. These results suggested that calcium from intracellular calcium storage may play an important role in quercetin-induced autophagy.

Key words: quercetin, autophagy, intracellular Ca\(^{2+}\), calcium chelator

Introduction

Autophagy is a kind of cellular catabolic degradation response to nutrient starvation or metabolic stress. And at the same time, autophagy delivers a fresh pool of amino acids and other essential molecules to cells [1]. Autophagy participates not only in maintaining cellular homeostasis, but also in promoting cell survival during cellular stress situations. Consequently, impaired or altered autophagy is often involved in several pathologies, such as cancer and neurodegenerative disorders. Most cells utilize Ca\(^{2+}\) influx from the extracellular space and Ca\(^{2+}\) release from intracellular stores to generate intracellular signals [2]. Intracellular Ca\(^{2+}\) is one of the regulators of autophagy. And the control of autophagy by intracellular Ca\(^{2+}\) signaling is the subject of two opposite views. On one hand, the available evidence indicated that intracellular Ca\(^{2+}\) signals, mainly inositol 1,4,5-trisphosphate receptors, suppress autophagy. On the other hand, elevated cytosolic Ca\(^{2+}\) concentrations were also shown to promote the autophagic process [3].

Flavonoids are ultraviolet (UV)-B absorbing secondary metabolites that are synthesized in higher plants, mosses, and ferns in order to protect them from the harmful effects of UV-B radiation and diseases [4]. Many studies have suggested that several flavonoid compounds such as rutin and quercetin have strong antioxidant and anti-proliferative activities [5,6]. Recently, quercetin has attracted considerable attention as a potential anti-cancer agent and an inducer of autophagy [7,8]. Quercetin, as one of the most common flavonoids in nature, has been extensively studied as a chemoprevention agent in several cancer models due to its potent antioxidant, anti-tumor, and anti-inflammatory properties. Compared with other flavonoids, quercetin has been demonstrated to be very effective in anti-tumor, antioxidant, and anti-inflammatory activities in the in vitro systems [6,9,10]. Quercetin can specifically target the PI3K/Akt pathway by competing with ATP binding, and efficiently inhibits cell survival, carcinogenesis, and tumor development, as well as the induction of apoptosis in vitro and in vivo [9,11–13]. Quercetin can also induce autophagy in MCF7 and gastric cancer cells by modulation of Akt/mammalian target of rapamycin (mTOR) signaling and hypoxia-induced factor-1α signaling [7].
However, whether intracellular Ca\(^{2+}\) can be induced by quercetin in HepG2 cells, and the role of intracellular Ca\(^{2+}\) in quercetin-induced autophagy progression is not clear. Therefore, this study was undertaken to examine the change of Ca\(^{2+}\) in this progress, and to explore their potential contribution to quercetin-induced autophagy so that the correlation between changes in [Ca\(^{2+}\)], and levels of autophagy can be elucidated. Solving the above problems would help to illustrate the mechanism by which the changes in intracellular [Ca\(^{2+}\)] in HepG2 cells induce autophagy and provide a theoretical basis on how the increase in intracellular Ca\(^{2+}\) may be a potent inducer of autophagy.

**Materials and Methods**

**Chemicals and reagents**

Ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), Fluo-3 acetoxyethyl (Fluo-3 AM) ester, and dimethyl sulfoxide (DMSO) were purchased from Solarbio Science & Technology Co. Ltd (Beijing, China). Monodansylcadaverine (MDC) was purchased from Sigma Aldrich (St Louis, USA). 1,2-Bis(2-aminophenoxyl)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl (BAPTA-AM) ester was purchased from Dojindo Laboratories (Shanghai, China). Cyto-ID™ Green Detection kit was purchased from Enzo Life Sciences (Farmingdale, USA). Antibodies were obtained from the following sources: anti-β-actin antibody (DA-1001; Invitrogen, Carlsbad, USA), anti-p62 and anti-LC3 antibodies (bs-2951R and bs-11731R; Bioss, Beijing, China), and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (81-6120; Invitrogen). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd (Hangzhou, China). All other chemicals and reagents used in the study were of analytical grade and were obtained from local chemical companies.

**Cell culture and treatment**

HepG2 human liver cancer cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HepG2 cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and in a 5% CO\(_2\) atmosphere. The cells were cultured in cell culture conditions 24 h prior to staining. The cells were suspended in 150 µl of phosphate buffer, and the supernatant was transferred to black 96-well plates. Fluorescence was detected by using a fluorometer. About 100 µl of quercetin was then added to each well and incubated for 30 min at 37°C. The cells were then washed with PBS twice. The coverslips with cells were infiltrated with 2–3 µl of PBS on microscope slides, and the cellular localization pattern of GFP-LC3 protein was then photographed using laser scanning confocal microscopy.

**Western blot analysis**

For western blot analysis, HepG2 cells were cultured in 6-well plates, and cell extracts were prepared by cell lysis in a radioimmunoprecipitation assay buffer. Cellular lysates were resolved on 10%–15% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, USA). The membranes were blocked with 4% non-fat milk powder in a buffer containing Tris–HCl (50 mM, pH 7.5), NaCl (150 mM), and Tween 20 (0.05%), and then incubated with the primary antibodies at 4°C overnight. The membranes were then washed and treated with the appropriate HRP-conjugated secondary antibodies for 4 h. The immunoreactivities were visualized using enhanced chemiluminescence reagents (CW0049; Cwbiootech, Beijing, China).

**Determination of the autophagic level**

The determination of autophagic level was conducted using a Cyto-ID™ Green Detection Reagent as previously described [15]. A total of 1 x 10\(^6\) cells per well were seeded in 6-well cell culture plates. The cells were treated with or without quercetin or co-incubated with EGTA or BAPTA-AM [16]. After the cells were incubated for 18 h, 1 µl of Cyto-ID™ Green Detection Reagent was dissolved to 4 ml as an assay buffer according to the manufacturer’s instruction. The cells in each well were incubated with 100 µl of diluted dye for 30 min at 37°C in the dark. The suspension was then centrifuged and harvested. The cells were then washed with PBS twice. Finally, the cells were re-suspended in 150 µl of phosphate buffer, and the fluorescence intensity was immediately measured with a flow cytometer. Excitation and emission wavelengths were 488 and 520 nm, respectively.

**Measurement of intracellular Ca\(^{2+}\) level**

Intracellular Ca\(^{2+}\) level were measured with a fluorescent spectrophotometer. About 100 µl of quercetin was then added to each well, and the concentrations of quercetin were 6.25, 12.5, 25, and 50 µg/ml. The blank control sample was 0.1% DMSO. After 10 min of treatment, the cells were washed with calcium-free buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM HEPES, 5 mM EGTA, pH 7.4). The cells were then stained with 1:5000 dilution of the cell permeable Ca\(^{2+}\) sensitive fluorescent dye Fluo-3 AM for 30 min in the dark [17]. The cells were washed with PBS three times. Finally, 100 µl ofphposphate buffer, and the fluorescence intensity was immediately measured with a flow cytometer. Excitation and emission wavelengths were 488 and 520 nm, respectively.

**Assessment of autophagy**

The assessment of autophagy was preliminarily conducted using MDC as previously described [14]. For organelle staining, 1 x 10\(^6\) cells per well were seeded on glass covered with slips in 6-well cell culture plates and grown in cell culture conditions 24 h prior to staining. After incubation with or without quercetin for 6 h, the culture medium was removed from the Petri dishes. The cells in each well were then co-incubated with 300 µl of 100 µM MDC solution in the cell culture medium for 30 min in the dark at 37°C. The cells were washed with phosphate buffer saline (PBS: 8.00 g/l NaCl, 0.20 g/l KCl, 0.2 g/l KH\(_2\)PO\(_4\), 1.56 g/l Na\(_2\)HPO\(_4\)-12H\(_2\)O) three times. The autophagy cells were observed by laser scanning confocal microscopy.

For transient transfection, the cells were transfected with pEGFP-LC3 plasmid by using a Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol and were maintained on coverslips in 6-well plates. After transfection for 24 h, the cells were treated with different concentrations of quercetin for 6 h. The cells were then washed with PBS twice. The coverslips with cells were infiltrated with 2–3 µl of PBS on microscope slides, and the cellular localization pattern of GFP-LC3 protein was then photographed using laser scanning confocal microscopy.
cytometer. Excitation and emission wavelengths were 488 and 525 nm, respectively.

To further measure the dynamic changes of [Ca^{2+}], laser scanning confocal microscopy was used to analyze the morphologic feature as previously described [18]. HepG2 cells were cultured in Petri dishes with a single well. First, the cells were co-incubated with Fluo-3 AM for 30 min at 37°C in the dark and then exposed to 50 µg/ml quercetin. Ten cells were randomly selected as targets to detect the dynamic changes in [Ca^{2+}], at the beginning of the experiments. The fluorescence of Ca^{2+} was detected by laser scanning confocal microscopy. The scanning number was 100, and the scanning time was 15 min. The raw fluorescent intensities (Fluo-3) of user-selected areas were used for data analyses.

Statistical analysis
Each experiment was repeated at least three times by using three independent samples per group. The results were expressed as the mean ± SE, and statistical analyses were performed by using SPSS16.0. *P < 0.05 was considered to be statistically significant.

Results
Quercetin-induced HepG2 cell autophagy
MDC is a specific autophagy marker that could be used to detect autophagic vacuoles [14]. The punctate structures in the cells imply the activation of autophagy. After the cells were exposed to 50 µg/ml quercetin for 6 h, autophagic vacuoles were observed. The results showed that the cells have more punctate structures after they were co-incubated with 50 µg/ml of quercetin for 6 h (Fig. 1A) compared with the control group, where fluorescence was dispersed in the cytoplasm. The results revealed that quercetin could significantly induce autophagy.

![Figure 1. Autophagy of HepG2 cells induced by quercetin](image)

Quercetin-induced [Ca^{2+}], elevation in a time- and dose-dependent manner
Considering the complex function of Ca^{2+}, the effect of quercetin on [Ca^{2+}], was explored. The cells were exposed to 0–50 µg/ml quercetin for 10 min, and [Ca^{2+}], was detected by using a spectrophotometer. The results showed that the level of [Ca^{2+}], in HepG2 cells was significantly increased when the concentration of quercetin was higher than 12.5 µg/ml (Fig. 3). This result implied that quercetin could induce the elevation of [Ca^{2+}], and is positively correlated with the concentration of quercetin. However, no significant change was observed between the control group and 0–12.5 µg/ml treatment group after the cells were treated for 10 min.

The effect of the quercetin treatment time on [Ca^{2+}], was also investigated. After the cells were exposed to 25–50 µg/ml quercetin for 10 min or 15 min, the average fluorescent intensity of [Ca^{2+}], was detected. The results showed that [Ca^{2+}], was significantly increased in HepG2 cells after treatment with quercetin for 10 min (Fig. 4A). The elevation of [Ca^{2+}], was positively correlated with quercetin concentration. However, [Ca^{2+}], was significantly decreased when the cells were treated for 15 min with 25 or 50 µg/ml quercetin, but [Ca^{2+}], remains a little higher than the control group (Fig. 4A).

Laser scanning confocal microscopy was performed to determine the instantaneous change in [Ca^{2+}], induced by quercetin and to determine the change in Ca^{2+}. Ten cells were randomly selected as targets to detect the dynamic changes in [Ca^{2+}], at the beginning of the experiments. The results showed that [Ca^{2+}], was slowly increased compared with the control group (Fig. 4B). The result revealed that the calcium from calcium storage was released into the cytoplasm when the cells were treated with quercetin. So the efflux of cytoplasmic calcium ions resulted in the transient increase of intracellular calcium levels. And the transient releases of calcium ions activated the autophagy pathways. This result further showed that the increased Ca^{2+} was primarily generated intracellularly.

Effects of EGTA and BAPTA-AM on cell autophagy induced by quercetin
In this study, BAPTA-AM and EGTA were used to detect the effect of intracellular or extracellular Ca^{2+} on the levels of autophagy induced...
by quercetin. When the HepG2 cells were incubated with quercetin or co-incubated with different concentration of BAPTA-AM for 18 h, the level of autophagy was determined. The results indicated that BAPTA-AM can effectively inhibit autophagy induced by quercetin as shown in Fig. 5A. BAPTA can inhibit quercetin-induced autophagy in a dose-dependent manner. After the cells were exposed to different concentrations of quercetin or co-incubated with EGTA and BAPTA-AM for 18 h, the level of autophagy was determined. The result showed that the fluorescence of quercetin + EGTA group was almost unchanged and that EGTA had no inhibitory effects on autophagy. Compared with the group treated with quercetin, the fluorescence of quercetin + BAPTA-AM group was decreased by 25.4 and 19.2%, respectively (quercetin: 25 or 50 µg/ml), which suggests that BAPTA-AM, as an intracellular Ca²⁺ chelator, inhibited cell autophagy induced by quercetin (Fig. 5B). EGTA is an extracellular Ca²⁺ chelator, whereas BAPTA-AM is an intracellular Ca²⁺ chelator.

Figure 2. Effects of quercetin on the autophagy of HepG2 cells (A) Western blot analysis of LC3 and p62 expressions in HepG2 cells treated with 25 or 50 µg/ml quercetin for 48 h. β-Actin was used as a loading control. (B) Relative expression of LC3-II and p62 were quantified by densitometry. (C) HepG2 cells were treated with 50 µg/ml quercetin, after 18 h culture, 100 µl diluted Cyto-ID™ Green Detection Reagent was added in the wells and incubated for 30 min at 37°C in the dark. The cells were then washed with PBS twice, and the fluorescence intensity was immediately measured with a flow cytometer. Excitation and emission wavelengths were 488 and 520 nm, respectively. *P< 0.05 and **P< 0.01 compared with the control group.

Figure 3. Assessment of quercetin-induced [Ca²⁺] i changes The cells were treated with different concentrations of quercetin as indicated. The average intensity of fluorescence was detected by flow cytometry. The [Ca²⁺] i of HepG2 increased in a dose-dependent manner after treatment with 6.25 or 50 µg/ml quercetin for 10 min. The effect of different concentrations of quercetin on [Ca²⁺] i was detected by fluorescent spectrophotometry. *P< 0.05 and **P< 0.01 compared with quercetin group.
EGTA has no effect on cell autophagy, but BAPTA-AM could inhibit the autophagy induced by quercetin. So we concluded that release of calcium by quercetin is from intracellular calcium storage that plays an important role in autophagy.

**Discussion**

Recently, quercetin has attracted considerable attention as a potential anti-cancer agent because of its effect on the apoptosis and autophagy in a variety of tumor cells [7,19]. Furthermore, quercetin induces autophagic processes and thereby reduces the half-life of oncogenic Ras protein, implying a novel chemopreventive mechanism associated with this flavonoid [20]. Quercetin was also suggested to induce apoptosis in gastric cancer cells by modulating Akt/mTOR signaling and hypoxia-induced factor-1α signaling, and thereby exhibited autophagy in a xenograft mouse model [7]. Meanwhile, competitive crosstalk between the ubiquitin-proteasome system and autophagy has also been recently reported. For example, when quercetin inhibited proteasomal activity, polyubiquitinated protein aggregates were accumulated and autophagy was increase via marked reduction in the phosphorylation of the mTOR substrates [21,22]. These results suggested that quercetin can be an autophagy inducer.
Calcium (Ca\(^{2+}\)) is a ubiquitous intracellular messenger that controls a diverse range of cellular processes, such as gene transcription, muscle contraction, and cell proliferation. Previous review has also described the dual functions of Ca\(^{2+}\) in autophagy regulation [3]. Høyer-Hansen et al. [23] reported that macroautophagy could be controlled by calcium and other calcium-related factors. Previous studies have been reported that calcium induced by quercetin is involved in some physiologic and pathological processes. Quercetin caused significant depletion of cytosolic calcium levels and decreased calcium uptake from the intracellular stores in MDA MB 231 cells [24]. Quercetin could stimulate insulin secretion by increasing Ca\(^{2+}\) flux through an interaction with L-type Ca\(^{2+}\) channels [25]. Natsume et al. [26] reported that quercetin suppressed the ER stress caused by calcium dynamics dysregulation by the inhibition of P38K. Quercetin may be useful in preventing inhibition of intestinal Ca\(^{2+}\) absorption caused by menadione or other substances that deplete glutathione leading to oxidative stress and apoptosis [27]. Quercetin may protect the GI mucosa against the adverse effects of NSAIDs by preventing mitochondrial dysfunction and by regulating intracellular Ca\(^{2+}\) homeostasis [6]. Cadmium-induced autophagy and apoptosis were also mediated by a calcium-signaling pathway, but quercetin manifested a protective effect against cadmium-induced cytotoxicity in rPT cells through inhibiting apoptosis, attenuating lipid peroxidation, and renewing mitochondrial function, followed by elevation of the non-enzymatic and enzymic antioxidants levels [28,29]. However, published results about the function of intracellular Ca\(^{2+}\) in autophagy induced by quercetin are still controversial [30]. In the present study, quercetin was found to induce autophagy and the elevation of [Ca\(^{2+}\)]. This elevation of [Ca\(^{2+}\)], was also changed in a concentration- and time-dependent manner (Figs. 3 and 4). These results demonstrated

Figure 5. Effects of EGTA and BAPTA-AM on quercetin-induced autophagy

(A) HepG2 cells were incubated with 50 µg/ml quercetin or co-incubated with different concentration of BAPTA-AM for 18 h, and then 100 µl diluted Cyto-ID™ Green Detection Reagent was added in the wells and incubated for 30 min at 37°C in the dark. The autophagic levels of cells were detected by flow cytometry. (B) HepG2 cells were exposed to different concentrations of quercetin, or incubated with EGTA (10 mM) and BAPTA-AM (5 µM), and then 100 µl diluted Cyto-ID™ Green Detection Reagent was added in the wells and incubated for 30 min at 37°C in the dark. The autophagic levels of cells were detected by flow cytometry. *P<0.05 and **P<0.01 compared with quercetin group.
that intracellular Ca\textsuperscript{2+} was elevated at the beginning of autophagy because of the induction of quercetin. Thus, we inferred that [Ca\textsuperscript{2+}], elevation appears to be a second messenger that causes autophagy induction. EGTA and BAPTA-AM were used to block extracellular Ca\textsuperscript{2+} and intracellular Ca\textsuperscript{2+}, respectively. BAPTA-AM treatment significantly suppressed quercetin-induced autophagy, whereas EGTA had no such inhibitory effects (Fig. 5B). The results demonstrated that the effect of extracellular Ca\textsuperscript{2+} on autophagy could be neglected. However, the reduction of [Ca\textsuperscript{2+}]\textsubscript{i} may suppress cell autophagy. Furthermore, our results suggested that the elevated [Ca\textsuperscript{2+}]\textsubscript{i}, induced by quercetin was mainly from the inside of cells. We surmised that the flow of calcium ions among cell organelles could induce autophagy.

Taken together, the results of this study demonstrated that quercetin functions as a small-molecule organic compound that can facilitate autophagy and [Ca\textsuperscript{2+}], elevation. Simultaneously, elevated Ca\textsuperscript{2+} was primarily sourced from the inside of cells and might be mediated by the release of Ca\textsuperscript{2+} from intracellular calcium storage but not by an influx of extracellular Ca\textsuperscript{2+}. Further research will focus on the specific mechanisms of quercetin-induced autophagy and [Ca\textsuperscript{2+}], elevation to reveal the calcium-signaling pathway of autophagy.

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**References**