Published in final edited form as:

Toxicol Appl Pharmacol. 2015 November 1; 288(3): 453–462. doi:10.1016/j.taap.2015.08.016.

Natural Products Induce a G Protein-Mediated Calcium Pathway Activating p53 in Cancer Cells

Paul R. van Ginkel^{1,2}, Michael B. Yan^{1,2}, Saswati Bhattacharya^{1,2,3}, Arthur S. Polans^{1,2,*}, and Jason D. Kenealey^{1,2,4}

¹UW Carbone Cancer Center, University of Wisconsin, Madison, WI 53792

²Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, WI 53792

³Department of Pediatrics, University of Wisconsin, Madison, WI 53792

⁴Department of Nutrition, Dietetics and Food Science, Brigham Young University, Provo, UT 84602

Abstract

Paclitaxel, etoposide, vincristine and doxorubicin are examples of natural products being used as chemotherapeutics but with adverse side effects that limit their therapeutic window. Natural products derived from plants and having low toxicity, such as quercetin, resveratrol, epigallocatechin gallate and piceatannol, have been shown to inhibit tumor cell growth both *in vitro* and in pre-clinical models of cancer, but their mechanisms of action have not been fully elucidated, thus restricting their use as prototypes for developing synthetic analogs with improved anti-cancer properties. We and others have demonstrated that one of the earliest and consistent events upon exposure of tumor cells to these less toxic natural products is a rise in cytoplasmic calcium, activating several pro-apoptotic pathways. We describe here a G protein/inositol 1,4,5-trisphosphate pathway (InsP3) in MDA-MB-231 human breast cancer cells that mediates between these less toxic natural products and the release of calcium from the endoplasmic reticulum. Further, we demonstrate that this elevation of intracellular calcium modulates p53 activity and the subsequent transcription of several pro-apoptotic genes encoding PIG8, CD95, PIDD, TP53INP, RRM2B, Noxa, p21 and PUMA. We conclude from our findings that less toxic natural products likely bind to a G protein coupled receptor that activates a G protein-mediated and calcium-dependent pathway resulting selectively in tumor cell death.

*To whom correspondence should be sent: Arthur S. Polans, Ph.D., Rm K6/466 Clinical Science Center, 600 Highland Avenue, University of Wisconsin, Madison, WI 53792. aspolans@wisc.edu, 608-265-4423.

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Conflict of Interest Statement

The authors disclose no potential conflicts of interest.

Author's Contributions

Experimental Design: P.R. van Ginkel, J.D. Kenealey and A.S. Polans

Data Acquisition and Analysis: P.R. van Ginkel, J.D. Kenealey, M.B. Yan, S. Bhattacharya and A.S. Polans

Writing and Reviewing: P.R. van Ginkel, A.S. Polans and J.D. Kenealey

Keywords

G-Protein Coupled Receptor; Calcium; Breast Tumor Cell Apoptosis

Introduction

Due to the toxic side effects of many chemotherapeutics more attention is being focused on less toxic natural products that still appear capable of inhibiting tumor cell growth *in vitro* and in pre-clinical models of cancer (for reviews see 1,2). Of the more promising natural products being investigated as anti-cancer agents, many are polyphenols, especially those derived from the family of flavonoids, including flavonols (i.e. quercetin), flavanols (i.e. EGCG) and isoflavones (i.e. genistein), as well as from the family of stilbenes (i.e. resveratrol and piceatannol). Such compounds have been shown *in vitro* to inhibit tumor cell proliferation and induce apoptosis across a broad spectrum of cancer cell types (1,3,4), as well as arrest tumor growth in pre-clinical studies through direct action on tumor cells (5,6) as well as the tumor microenvironment (7). While their bioavailability may be limited *in vivo*, these compounds nonetheless have significant *inhibitory* capacity and are capable of instigating tumor *regression* when their bioavailability is increased (8,9). Of significance, under these circumstances there are no detrimental consequences for normal cells and tissues (8).

These less toxic flavonoids and stilbenes have been shown to activate numerous and diverse cellular pathways that can affect the development and progression of tumors (for reviews see 3,10). Some of these pathways are activated within a matter of seconds, while others transpire over hours or days, making it difficult to distinguish between events activated as a consequence of direct binding by the natural product versus the multitude of indirect events occurring downstream of the initial target interaction. Resolution of the issue has been further complicated by the erroneous identification of putative target molecules (11). One of the earliest and consistent events observed in tumor cells *in vitro* following the addition of quercetin (12), resveratrol (13,14), EGCG (15), piceatannol (16) and similar products (17,18) is an increase in intracellular calcium. Calcium is perhaps the most ubiquitous and versatile second messenger in biology. Its levels are strictly regulated in the cytoplasm, where concentrations greater than 10^{-5} M are toxic (for review see 19). As a result of both exclusion and sequestration, calcium gradients can be put to work to form complex signals that are sensed by an array of calcium-responsive proteins in the cell (20).

Calcium-mediated signaling by extrinsic factors can be initiated via different receptors, prominent among them the GPCRs and the RTKs. Binding of ligand to a GPCR can lead to the activation of PLC, mediated through a G-protein, whereas binding to an RTK generally leads to intracellular receptor binding of SH2 domain-containing proteins and adaptor proteins that result in the activation of PLC. PLC in turn cleaves PIP₂, producing InsP₃ which then binds to its receptor (InsP₃R) and opens a channel releasing calcium from the ER.

Considerable evidence demonstrates that the release of calcium from the ER and its subsequent accumulation in the mitochondria can activate cell death (21–23). The uptake of

calcium by the mitochondria triggers the permeability transition pore, the opening of which is associated with mitochondrial depolarization and the release of small proteins such as cytochrome c and SMAC/DIABLO (23). In turn these small proteins induce the formation of the so-called apoptosome and subsequently the activation of a caspase cascade (24). We have shown that this “intrinsic” apoptotic pathway is one conduit to tumor cell death activated by several of the less toxic natural products (8,13). An increase in cytoplasmic calcium also activates an additional family of cysteine proteases, the calpains, which among other functions degrade pumps and exchangers involved in maintaining calcium homeostasis, again leading to cell death (13). The depletion of ER calcium as a consequence of treatment with less toxic flavonoids and stilbenes also can instigate an ER stress response (25–27). Finally, the activity of several transcription factors, including NF κ B and NFAT, is regulated by calcium (28,29), affecting tumor development and progression (3). Therefore, the final outcome of exposing tumor cells to less toxic natural products is a multi-faceted calcium response leading to tumor cell death through a combination of parallel pathways. For these reasons, it may be possible to utilize calcium signaling to activate tumor cell death as a novel mechanism underlying safe and effective chemotherapeutic intervention.

To further define the calcium pathway in tumor cells initiated by less toxic flavonoids and stilbenes, we describe a combination of molecular and pharmacological experiments demonstrating the involvement of a GPCR, PLC, and InsP3 acting upon InsP3R to release ER calcium. We also demonstrate that cAMP has no significant role in activating these calcium signals in tumor cells, as might be suggested from other studies, and therefore that adenylate cyclase (30) and cAMP-PdE (31) are unlikely to be the direct targets of these natural products.

In addition to published observations from our laboratory and others that natural products cause tumor cell death by activating the intrinsic apoptotic pathway (8), the family of calpains (13) an ER stress response (25) and certain transcription factors (3), we also demonstrate here that less toxic plant flavonoids and stilbenes cause a calcium-dependent increase in the activity of p53, a central regulator of cell proliferation and cell death, and a major target of mutational events in solid tumors. We believe that the cumulative effect of activating multiple calcium-dependent pathways contributes to the efficacy of these compounds at causing tumor cell death in a wide variety of cancers.

Methods

Materials

The MDA-MB-231 cell line was purchased from the American Type Culture Collection. Resveratrol was obtained from Cayman Chemical (Ann Arbor, MI). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose, L-glutamine and sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic/antimycotic solution (Invitrogen, Grand Island, NY). Cultures were maintained at 37°C in 95% O₂/5% CO₂.

Fura-2-AM was supplied by Molecular Probes (Grand Island, NY). M119K was obtained through the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program,

National Cancer Institute. BAPTA-AM and GPCR Helix 8 Signaling Inhibitor JF5 came from Calbiochem (Billerica, MA). $G_{\alpha q/11}$ goat polyclonal antibody and rabbit antibodies specific for $G_{\beta 1}$ and $G_{\beta 2}$ were obtained from Santa Cruz Biotechnology (Dallas, TX). GAPDH mouse monoclonal antibody was purchased from Biogenesis (Raleigh, NC). Rabbit antibodies specific for p53 and phospho (S15) p53 were purchased from Cell Signaling Technology (Danvers, MA). $G_{\alpha q/11}$, $G_{\alpha 15}$, $G_{\beta 1}$, $G_{\beta 2}$, GAPDH and non-targeting siRNAs as well as Dharmafect 4 were obtained from Dharmacon (Lafayette, CO). All other reagents were procured from Sigma Chemical Company (St. Louis, MO).

Intracellular Calcium Imaging

Changes in $[Ca^{2+}]_i$ were determined using fura-2-AM, as described previously (13). Briefly, 13,000 cells per well were plated in 96-well imaging plates (BD Biosciences, San Jose, CA) two days prior to calcium imaging. After reaching 80–90% confluence, the cells were loaded with fura 2-AM by incubation in Hank's Balanced Salt Solution (HBSS; Invitrogen) with 3 mg/ml bovine serum albumin and 15 μ M fura-2-AM at 37°C for 45 min. The solution was then replaced with HBSS and incubation continued for 30 min at room temperature to allow for hydrolysis of the AM-ester. Stock solutions of each natural product in DMSO were diluted in HBSS. Pre-incubations with different pharmacological agents were conducted for 30 min prior to the addition of the natural product. Serial acquisitions of fluorescent images of cells were performed in a non-confocal mode on a BD Pathway Bioimager (BD Biosciences) using a 20 $\times$ UApo340 objective. Fura-2-AM was alternately excited at wavelengths of 340 and 380 nm, and emitted fluorescence was collected at 510 nm. Images were collected at 4 s intervals for 40 s before the addition of drugs and image collection continued for at least 300 seconds thereafter. Subsequently, $[Ca^{2+}]_i$ maps for regions of interest (ROIs) which contained between 1 and 5 cells (about 5–10 ROIs per viewing field) were calculated using Attovision 5.0 three-dimensional imaging software. Overlapping intervals of 4 time points were averaged. The standard error was calculated for 340/380 ratios of the cells in a viewing area at each measuring point. Statistical comparisons were performed using the unpaired Student's t-test.

Cell Transfection

Cells were plated at 5,500 cells per well in a 96-well imaging plate one day prior to transfection. siRNAs were diluted to 0.25 μ M in OptiMEM (Invitrogen) and Dharmafect 4 at 1/100 in OptiMEM. Equal volumes of the solutions were incubated together for 30 min at room temperature, diluted with 4 $\times$ volume antibiotic free medium and plated onto the cells. Cells were transfected for 72 hours followed by calcium imaging. Additional sets of wells were transfected for Western analysis of targeted proteins.

Calcium-dependent p53 Expression

Cells were plated at 500,000 cells per dish onto 60 mm dishes two days prior to the addition of natural products. After reaching 80–90% confluence, cells were pre-incubated for 30 minutes with 20 μ M BAPTA-AM or 0.1% DMSO. After washing the cells to remove any excess BAPTA-AM, one hundred μ M of each natural product was then added to the plates and incubation was continued for 2 hours. Cells were collected following treatment with trypsin/EDTA, washed with HBSS and lysed in SDS sample buffer with protease and

phosphatase inhibitors for Western analysis as described (13). The analysis of p53-dependent gene expression by quantitative PCR followed the treatment of cells for 6h with 100 μ M resveratrol or 0.1% DMSO, collection by trypsin/EDTA and lysis in RLT buffer (Qiagen, Valencia, CA).

Quantitative PCR

Following homogenization of the cell lysates using Qiashredder columns (Qiagen) and removal of genomic DNA with a gDNA column (Qiagen), RNA was isolated from drug- and control-treated cells using an RNeasy mini kit (Qiagen) and reverse transcribed using the first strand cDNA synthesis system (Invitrogen). Quantitative PCR was performed with SYBR mix (BioRad, Hercules, CA) using PIG8, CD95, PIDD, TP53INP, RRM2B, Noxa, p21 and PUMA-specific primers (Supplementary Table S1) and POLR2A-specific primers (Supplementary Table S1) as an internal standard using an iCycler (BioRad) according to the manufacturer's instructions. All reactions were performed in triplicate, and the experiment was repeated three times. Relative expression levels and standard deviations were calculated using the comparative method (Applied Biosystems, Grand Island, NY).

cAMP Assay

The cyclic AMP complete enzyme immunoassay kit from Assay Designs (Farmingdale, NY) was used according to the manufacturer's directions utilizing 2×10^6 cells per sample.

Transcriptome

The preparation of MDA-MB-231 cells for sequencing and the Illumina RNA-Seq runs on a Genome Analyzer II in the Genomics Core Facility at Case Western Reserve University were performed according to published procedures (32). Illumina software was used to calculate normalized levels of expression.

Results

The excessive accumulation of calcium in the cell cytoplasm, either by entry through channels in the plasma membrane or by release from internal stores, has been shown to activate apoptotic pathways in diverse cell types both during normal development and as a consequence of certain pathologies. Our long-term objective is to determine whether such calcium signaling can be utilized to activate tumor cell death as a basis for safe and effective chemotherapeutic intervention. We demonstrate in Figure 1A that less toxic natural products elicit a rapid rise in cytoplasmic calcium in MDA-MB-231 breast cancer cells loaded with the calcium-sensitive reporter fura-2. The potential chemopreventive and chemotherapeutic natural products quercetin, resveratrol, EGCG, and piceatannol initiated a rise in cytoplasmic calcium within 30 seconds of their addition. In contrast, no alteration in the concentration of cytoplasmic calcium was observed following treatment with etoposide and doxorubicin, also natural products but with adverse side effects when employed as chemotherapeutics. The synthetic chemotherapeutic cyclophosphamide also failed to elicit a change in the cytoplasmic concentration of calcium.

As shown in Figure 1A, the shape of the calcium response differs between flavonoids and stilbenes. The flavonoids like quercetin and EGCG, caused a monophasic response, whereby the peak calcium response was followed by a relaxation to baseline. However, stilbenes like resveratrol and piceatannol typically elicited a biphasic response; an initial peak that partially relaxed and then was followed by a gradual secondary increase.

Calcium signaling has been shown to be critical to the anti-cancer properties of these less toxic natural products (13,14). We demonstrate here that calcium signaling stimulated by less toxic flavonoids and stilbenes also modulates the activity of p53, an essential regulator of apoptosis and a vital tumor suppressor. Figure 1B shows Western blots stained for p53 and phospho (S15) p53 in MDA-MB-231 breast cancer cells following their treatment either with quercetin, resveratrol, EGCG or piceatannol. The levels of p53 protein increased with the addition of each natural product compared to the DMSO control and the phosphorylation of p53 increased concomitantly. However, a brief pre-incubation of cells with BAPTA-AM for intracellular incorporation, followed by the removal of any excess BAPTA-AM from the medium, blocked the rise in p53 protein levels and phosphorylation. To verify the effect, RNA was isolated from MDA-MB-231 cells following treatment with resveratrol and quantitative PCR was performed using primers directed to known p53-dependent genes (Figure 1C). The activation of a variety of pro-apoptotic genes was observed.

Since calcium signaling is important to the anti-cancer activity of several less toxic flavonoids and stilbenes, we proceeded to delineate the cellular pathway mediating between these products and the final rise in cytoplasmic calcium. Figures 2A–C illustrate that the less toxic natural products activate the same initial source of calcium. As shown in Figure 2A, treatment with quercetin produced a stereotypic monophasic response. If EGCG was subsequently added to the same cells, there was no further increase in cytoplasmic calcium, indicating that quercetin likely had depleted the source of calcium necessary for an EGCG response. Also as shown in Figure 2A, if resveratrol treatment followed the addition of quercetin then the initial phase of the resveratrol response was absent, however the second, prolonged phase was still activated. These data indicate that quercetin and resveratrol share the same initial source of calcium, but resveratrol may be capable of activating a second store of calcium not normally accessed by quercetin (discussed below). Figures 2B and 2C demonstrate that the order in which these natural products are added does not affect the outcome. In each case the monophasic calcium responses associated with quercetin and EGCG as well as the first phase of the resveratrol response appeared to arise from the same store of calcium.

To determine the source(s) of calcium evoked by these natural products, MDA-MB-231 cells were pre-incubated with thapsigargin, an inhibitor of the ER calcium pump. After an initial rise in cytoplasmic calcium owing to treatment with thapsigargin, quercetin was added to the same cells (Figure 2D). The pre-treatment with thapsigargin abolished the response to quercetin. In a parallel experiment the initial phase of the calcium response to resveratrol also was eliminated by pre-treatment with thapsigargin, while the second, slower phase remained unaffected (Figure 2D). Similarly, thapsigargin abolished the response to EGCG and piceatannol (not shown). The data indicate that these natural products likely cause the release of calcium from the ER.

As reported by others (33), a portion of the calcium response may be contingent upon extracellular calcium. Therefore, the responses to quercetin and resveratrol were measured in the presence and absence of extracellular calcium. Supplementary Figures S1A and S1B illustrate that removal of extracellular calcium coupled with the addition of 50 μ M EGTA partially reduced the calcium response to both quercetin and resveratrol. In the absence of extracellular calcium, the responses to quercetin and resveratrol were reduced by 39% and 33%, respectively. These findings indicate that the rise in cytoplasmic calcium induced by these less toxic natural products is dependent on calcium stores derived from the ER as well as a contribution either directly or indirectly from the extracellular milieu.

To further define the pathway intervening between less toxic natural products and the release of calcium from the ER, a series of molecular and pharmacological experiments were performed. When MDA-MB-231 cells were treated with the GPCR-helix 8 signaling inhibitor JF5 (34), the response to quercetin was abolished as illustrated in Figure 3A, suggesting that signaling might be occurring through a GPCR. Since JF5 can selectively block G-protein signaling mediated through $G_{\alpha q}$, we focused initially on the G_{α} subunits q, 11 and 15 as well as the G_{β} subunits 1 and 2. An analysis of the MDA-MB-231 cell transcriptome revealed that these G_{α} and G_{β} subunits are expressed at significant levels among the principle isoforms of G_{α} and G_{β} in these cells (Supplementary Table S2). To support the pharmacological observation utilizing JF5 that suggests the involvement of a G protein-mediated pathway, MDA-MB-231 cells were transfected with siRNAs specific to the q/11 and 15 isoforms of G_{α} and the 1 and 2 isoforms of G_{β} . Various transfection combinations were tested. Only siRNA specific to $G_{\alpha q/11}$ combined with siRNA specific to $G_{\beta 1}$ and $G_{\beta 2}$ was capable of suppressing the calcium response in MDA-MB-231 cells exposed to quercetin (Figure 3B, gray line). Non-targeting siRNAs used as a control in the same experiment had no effect on the calcium response (Figure 3B, black line). Similar results were obtained following treatment with resveratrol (Figure 3C). siRNA targeting individual α or β subunits alone had little effect on calcium signaling nor did targeting $G_{\alpha 15}$. To further support a role for G_{β} in the signaling pathway, cells were pre-incubated with M119K, a $G_{\beta \gamma}$ inhibitor (35), prior to the addition of quercetin (Figure 3D). Under these conditions M119K blocked the calcium response. Collectively, these data support a model whereby a G protein is activated by less toxic natural products as part of the pathway underlying calcium signaling in MDA-MB-231 cells.

Since PLC often intercedes in the activation of ER calcium via a GPCR/G protein pathway, MDA-MB-231 cells were pre-incubated with vehicle alone or U73122, an inhibitor of PLC, and then treated with quercetin, resveratrol, EGCG or piceatannol (Figures 4A–D). In each case the results demonstrate that the PLC inhibitor suppressed the calcium responses to these compounds. As an additional control, pre-incubation with the inactive PLC inhibitor U73433 had no effect on the increase of cytoplasmic calcium (Figure 4A, light gray line). Consistent with these findings, pre-incubation of MDA-MB-231 cells with 2-APB, an inhibitor of the InsP3R, blocked the calcium response to quercetin and EGCG (Figures 4E,G) as well as the initial phase of the calcium response to resveratrol and piceatannol (Figure 4F,H). Expression of the ryanodine receptor was not detected in the transcriptome analysis of MDA-MB-231 cells, and the calcium responses to quercetin and resveratrol were unaffected by pre-treatment of cells with ryanodine and ruthenium red, two inhibitors of the

ryanodine receptor (data not shown). Since it has been reported that 2-APB also can inhibit store-operated channels in the plasma membrane in some cell types, we tested an inhibitor specific to this type of channel. The responses were unaffected by pre-incubation with SKF96365 (Supplementary Figures S2E and S2F). Collectively, the data support the model that less toxic natural products activate PLC causing an increase in InsP3, which in turn instigates the release of ER calcium through the InsP3R.

To address the possibility that cAMP might become elevated upon exposure to less toxic natural products and underlie the rise in cytoplasmic calcium observed in our experiments (30,31), cAMP levels were measured in MDA-MB-231 cells by direct immunoassay. No significant differences were detected between cells treated with DMSO and resveratrol (DMSO: 3.5 ± 0.8 pmoles cAMP; resveratrol: 3.8 ± 0.4 pmoles cAMP). Pre-incubation with IBMX to inhibit PdE activity increased basal levels of cAMP 30–40%, but the addition of resveratrol had no further effect (IBMX: 5.1 ± 1.8 pmoles cAMP; IBMX/resveratrol: 5.8 ± 1.1 pmoles cAMP). We pursued this line of investigation further using pharmacological approaches to test whether natural products activate calcium by elevating cAMP through direct interaction with either adenylate cyclase or cAMP-PdE. MDA-MB-231 cells were pre-incubated with SQ22356 and MDL12,330A, inhibitors of adenylate cyclase and PdE, respectively; neither interfered with the calcium responses to quercetin or resveratrol (Supplementary Figures S3A–D). Together, these experiments demonstrate that cAMP is unlikely to regulate the rise in cytoplasmic calcium in MDA-MB-231 cells induced by less toxic natural products. Rather, the data presented in this study support the likelihood that less toxic flavonoids and stilbenes elicit a calcium response in tumor cells through the activation of a GPCR (Figure 5).

Discussion

Alterations in cellular pathways affecting cell proliferation, apoptosis and migration can underlie cancer growth and metastasis, and these pathways often are modulated by calcium. Since less toxic natural products can activate calcium signals in different types of cancer cells (13–18,36) these compounds may be useful prototypes for the development of new, calcium-based chemotherapeutics that remain safe while effective.

To optimize the design of such chemotherapeutics, it would be useful to understand better the mechanisms of action of these less toxic natural products. The data presented in this paper are consistent with such products causing the release of ER calcium through a G protein/InsP3-dependent pathway. Both molecular and pharmacological approaches support the involvement of $G_{\alpha_q/11}$ coupled either with G_{β_1} or G_{β_2} in mediating between these natural products and the release of ER calcium. Consistent with these findings, we conducted preliminary experiments showing that calcium signaling was unaffected by the pre-treatment of MDA-MB-231 cells with pertussis toxin, thereby likely excluding roles for G_s or G_i . In addition, PLC ϵ was not detected in our transcriptome analysis of MDA-MB-231 cells, likely excluding $G_{\alpha_{12/13}}$ from participating in the calcium response.

The activation of $G_{\alpha_q/11}$ by other stimulants such as vasopressin, angiotensin, serotonin, epinephrine, and acetylcholine often triggers PLC, followed by the production of InsP3 and

the subsequent release of ER calcium through the InsP3R (37). Experiments presented here demonstrate that less toxic natural products can activate the same pathway. Our experiments suggest that a subtype of GPCR with a constrained helix 8 may initiate the calcium signaling. Activation of the GPCR is thought to then lead to GDP/GTP exchange on the alpha subunit of the bound heterotrimeric G protein causing the dissociation of the alpha subunit, which then directly activates PLC. The effect of JF5, which inhibits signaling through the $G_{\alpha q/11}$ subunits, as well as our knockdown of $G_{\alpha q/11}$ demonstrate the involvement of these alpha subunits in the release of intracellular calcium in response to less toxic natural products. In addition we demonstrate that the $G_{\alpha q/11}$ subunit of the heterotrimeric G protein can complex either with $G_{\beta 1}$ or $G_{\beta 2}$ to attain this activity. Alpha as well as beta/gamma subunits have been shown capable of activating PLC directly. In addition to $G_{\alpha q/11}$ the involvement of a beta subunit during the activation of PLC is supported in our studies by the inhibition of calcium signaling by M119K. PLC involvement in this signaling cascade was demonstrated further using the inhibitor U73122. Efforts to narrow the PLC activity to individual PLC beta, gamma or delta family members using siRNA approaches were unsuccessful, possibly indicating redundancy among multiple subfamily members. Finally, we demonstrate that release of calcium from the ER occurs via the InsP3R.

A recent study concluded that resveratrol elevates levels of cAMP in C2C12 myotubes, which then leads to an increase in intracellular calcium (31). An additional study also reported that resveratrol causes an increase in cAMP in MCF-7 tumor cells (30). The two studies identified different underlying targets as responsible for the rise in cAMP, the direct inhibition of cAMP-PdE-4 by resveratrol (31) and the activation of adenylate cyclase by resveratrol (30). We found no evidence that cAMP, through activation of adenylate cyclase or the inhibition of PdE, contributes to the calcium response in MDA-MB-231 tumor cells. Nonetheless, these putative targets may be essential in other cell types, wherein their activation may be valuable towards the prevention and/or treatment of diseases other than cancer. It's also important to note that while our studies focus on calcium-dependent cellular events, other important pathways are activated by less toxic natural products (3). It's the combination of these calcium-dependent and independent events that ultimately determines the cellular outcome.

To further elucidate the calcium signaling pathway initiated in tumor cells by less toxic natural products, it will be essential to identify the putative GPCR which directly binds these products. Once identified, the target can be used to screen for additional natural products or synthetics with potentially improved therapeutic properties. The precise binding site also can be determined, since there is no information about whether the natural products described in the current study bind to the normal ligand binding domain of a GPCR or some other site, perhaps involved in the direct binding of the G protein. Once the GPCR is identified, further studies also could elucidate the structural elements required of the natural products for activation of the target and subsequent calcium signaling. Of further significance, the expression levels of the target can be compared between cancer and normal cells. Reduced expression of the target in normal cells, for example, might help explain the differential cytotoxicity of these natural products observed in numerous animal and human studies (5,6).

Finally, if differentially expressed, the target itself could become a site for alternative chemical or biological intervention.

In this paper we demonstrate that less toxic natural products activate p53, increasing both protein levels and phosphorylation. We further demonstrate for the first time that in MDA-MB-231 tumor cells this activation is calcium-dependent. In separate experiments we did not observe an increase in mRNA encoding p53 upon treatment of MDA-MB-231 cells. Therefore, the elevation of p53 observed in the current study is likely due to changes in protein stability and the rate of proteosomal processing. Several S100 calcium binding proteins have been shown to modulate p53 protein stability in response to calcium (38,39); whether this occurs in MDA-MB-231 cells following the rise in cytoplasmic calcium instigated by less toxic natural products remains to be determined. Resveratrol also has been shown to bind to G3BP1, leading to the deubiquitination of p53 and thereby its elevation in the cell (40). It's not yet known whether calcium plays a role in this process.

During development and in response to genotoxic and non-genotoxic stress signals, the activation of p53 leads to transcriptional events, the consequence of which is growth arrest followed either by cellular repair or apoptosis. Here, we demonstrate the induction of several p53-dependent pro-apoptotic genes as a result of treatment with less toxic natural products.

Considering the variety of calcium-mediated, pro-apoptotic pathways activated by less toxic natural products, such compounds might well serve as prototypes for the development of a new class of chemotherapeutic agent, calcium mimetics, which utilize pre-existing, pro-apoptotic calcium signaling pathways to selectively eliminate tumor cells. Although less toxic natural products have low bioavailability due to marginal uptake in the gastrointestinal tract and rapid metabolism, they nonetheless inhibit tumor growth in numerous mouse models of cancer (3). When their bioavailability is enhanced by peri- or intra-tumor injection, thus bypassing the gut and reducing first-pass metabolism to inactive forms (8), tumor regression can be achieved with no adverse side effects (8,9). Therefore, synthetic modifications that decrease metabolism and increase solubility for controlled intravenous delivery could improve the bioavailability and efficacy of novel calcium mimetics.

The need exists for new anti-cancer agents with greater efficacy and a wider therapeutic window. While more toxic natural products are a mainstay in current cancer treatment, the study of less toxic natural products is revealing alternative mechanisms of action that may be utilized to design new drugs, especially for combined therapies involving non-overlapping biochemical interactions to elicit the optimum anti-tumor effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Drs. Debarshi Mustafi and Krzysztof Palczewski at Case Western Reserve University for conducting the transcriptome analysis described in this paper. We also wish to thank Chad Smith for technical support in the preparation of the manuscript.

Grant Support

Support was provided by the Retina Research Foundation (RRF). ASP is the recipient of the Kathryn & Latimer Murfee RRF Chair. Further support was provided by NIH grant P30 EY016665 and a grant from Research to Prevent Blindness to the Department of Ophthalmology and Visual Sciences.

Abbreviations

EGCG	epigallocatechin gallate
GPCRs	G-protein coupled receptors
RTKs	receptor tyrosine kinases
PLC	phospholipase C
PIP2	phosphatidylinositol 4,5-bisphosphate
InsP3	inositol 1,4,5-trisphosphate
InsP3R	inositol 1,4,5-trisphosphate receptor
ER	endoplasmic reticulum
cAMP-PdE	cAMP-phosphodiesterase
M119K	8-4,5,6-trihydroxy-3-oxo-3 <i>H</i> -xanthen-9-yl-1-naph-thalene-carboxylic acid
G3BP1	Ras-GTPase-activating protein SH3 domain-binding protein 1

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Highlights

Natural products having low toxicity increase cytoplasmic calcium in cancer cells.

A G-protein/IP₃ pathway mediates the release of calcium from the ER.

The elevation of intracellular calcium modulates p53 activity.

p53 and other Ca²⁺-dependent pro-apoptotic pathways inhibit cancer cell growth.

Figure 1.

Non-toxic natural products instigate an increase in $[Ca^{2+}]_i$ which activates p53. **A.** Fura-2 loaded MDA-MB-231 cells were alternately excited at 340 and 380 nm and emission was monitored at 510 nm. Following 40 seconds of baseline data collection, either 100 μ M quercetin, 100 μ M resveratrol, 50 μ M EGCG, 100 μ M piceatannol, 50 μ M etoposide, 2 mg/ml doxorubicin or 700 μ M cyclophosphamide was added to the cells. Plotted are the average changes in fluorescence intensity ratio (F_{340}/F_{380}) of the Ca^{2+} bound/ Ca^{2+} free fura-2 over time. **B.** p53 and phospho-p53 were measured by Western analysis in whole cell

lysates of MDA-MB-231 cells treated for 2 h with 100 μ M of resveratrol, quercetin, EGCG or piceatannol in the presence or absence of 20 μ M BAPTA-AM. GAPDH served as a loading control. **C.** p53-dependent gene expression was analyzed by quantitative PCR in MDA-MB-231 cells treated for 6 h with 100 μ M resveratrol and compared to control-treated cells. Expression was normalized to PolR2A levels. Measurements were performed in triplicate and plotted as mean $\pm$ standard deviation. Data represent one of three independent experiments.

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Figure 2.

Less toxic natural products mobilize the same source of ER calcium. In each experiment either quercetin (Q), EGCG (E), resveratrol (R) or thapsigargin (T) is added at time zero and the calcium response is monitored as described in the legend to Figure 1. The arrowhead in each panel then indicates when a second compound is added and the calcium response continues to be measured. **A.** Calcium responses to the sequential addition of 100 μ M quercetin followed at 450 s (arrowhead) by 50 μ M EGCG (blue line) or 100 μ M resveratrol (red line). **B.** Calcium responses to the sequential addition of 50 μ M EGCG followed at 450 s (arrowhead) by 100 μ M resveratrol (red line) or 100 μ M quercetin (blue line). **C.** Calcium responses to the sequential addition of 100 μ M resveratrol followed at 350 s (arrowhead) by 50 μ M EGCG (blue line) or 100 μ M quercetin (red line). **D.** The role of ER calcium in the response to less toxic natural products was measured by the sequential addition of 10 μ M thapsigargin followed at 350 s (arrowhead) by 100 μ M quercetin (blue line) or 100 μ M resveratrol (red line). Standard error was calculated for the F340/F380 measurements of the ROIs at each data point.

Figure 3.

The G protein subunits $G_{\alpha q/11}$ and $G_{\beta 1}$ or $G_{\beta 2}$ mediate calcium signaling by less toxic natural products. Calcium signaling in MDA-MB-231 was measured as described in Figure 1. The average F_{340}/F_{380} over time is plotted. **A.** Calcium responses to 100 μ M quercetin of cells pre-incubated with vehicle (black line) or 50 μ M JF5 (gray line). **B.** Calcium responses to 100 μ M quercetin in cells co-transfected with non-targeting siRNA (black line) or with $G_{\alpha q/11}$ and $G_{\beta 1}$ and $G_{\beta 2}$ siRNAs (gray line). Inset shows knockdown of $G_{\alpha q/11}$, $G_{\beta 1}$ and $G_{\beta 2}$ by Western analysis of G protein siRNA and control siRNA transfected cells and GAPDH as a loading control. **C.** Calcium responses to 100 μ M resveratrol in cells co-transfected with non-targeting siRNA controls (black line) or with $G_{\alpha q/11}$, $G_{\beta 1}$ and $G_{\beta 2}$ siRNAs (gray line). **D.** Calcium responses to 100 μ M quercetin of cells pre-incubated with vehicle (black line) or with 50 μ M M119K (gray line). Standard error was calculated for the F_{340}/F_{380} measurements of the ROIs at each data point.

Figure 4.

Less toxic natural products activate PLC leading to release of ER calcium through the InsP3R. Calcium signaling in MDA-MB-231 was measured as described in Figure 1. The average F_{340}/F_{380} over time is plotted. Calcium response to 100 μ M quercetin (A), 100 μ M resveratrol (B), 50 μ M EGCG (C) and 100 μ M piceatannol (D) in cells pre-incubated with vehicle (black lines) or 5 μ M U73122 (dark gray lines). 5 μ M U73433 was used as an inactive inhibitor control (A, light gray line). Calcium responses to 100 μ M quercetin (E), 100 μ M resveratrol (F), 50 μ M EGCG (G) and 100 μ M piceatannol (H) in cells pre-

incubated with vehicle (black lines) or 100 μ M 2-APB (gray lines). Standard error was calculated for the F340/F380 measurements of the ROIs at each data point.

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Figure 5.

Modeling the calcium-mediated pro-apoptotic action of less toxic natural products. **A.** Less toxic natural products target a constrained helix 8 containing GPCR leading to the activation of a G protein. The G $\alpha_{q/11}$ as well as the G β_1 and G β_2 subunits of the heterotrimeric G protein are released and activate PLC which results in the cleavage of PIP₂ into InsP₃ and DAG. InsP₃ binds to the InsP₃R, a Ca²⁺-channel in the ER membrane, leading to release of ER calcium into the cytoplasm. **B.** The rise in [Ca²⁺]_i leads to activation of the intrinsic apoptotic pathway, by uptake of Ca²⁺ into the mitochondria, opening the mPTP and

releasing SMAC/DIABLO and cytochrome c from the mitochondria and binding to XIAP and APAF1, respectively as part of the apoptosome. This leads to the activation of caspase 9 which in turn activates caspase 3 resulting in DNA fragmentation and apoptosis. Activation of calpains, Ca^{2+} activated endoproteases, leads to cleavage of calcium pumps (e.g. PMCA1), cytostructural proteins (e.g. fodrin) and nuclear substrates, resulting in the breakdown of cellular architecture and apoptosis. A lowering of ER calcium levels results in the unfolded protein response (UPR) due to the negative effect on Ca^{2+} -dependent molecular chaperones such as GRP78. This is followed by activation of the signaling protein PERK which phosphorylates eIF2 α and leads to the shutoff of mRNA translation except for certain transcripts like ATF4. This transcription factor activates the promotor of DDIT-3 (CHOP). DDIT-3 regulates additional genes leading to apoptosis. The increase in cytoplasmic calcium also leads to the activation of p53 and multiple pro-apoptotic gene products in different cellular compartments. Apoptosis is further enhanced by crosstalk between the aforementioned pathways.