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Natural products induce a G protein-mediated calcium pathway activating p53 in cancer cells☆

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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.

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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 Gupta et al., 2010; Tan et al., 2011). 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 (Gupta et al., 2010; Aggarwal and Shishodia, 2006; Piotrowska et al., 2012), as well as arrest tumor growth in pre-clinical studies through direct action on tumor cells (Bishayee, 2009; Hu et al., 2014) as well as the tumor microenvironment (Sagar et al., 2006). 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 (Van Ginkel et al., 2007; Soto et al., 2011). Of significance, under these circumstances there are no detrimental consequences for normal cells and tissues (Van Ginkel et al., 2007).

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 (Aggarwal and Shishodia, 2006; Pirola and Fröjdö, 2008)). 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 (Subramanian et al., 2010). One of the earliest and consistent events observed in tumor cells

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*H*-xanthen-9-yl)-1-naph-thalene-carboxylic acid); G3BP1, Ras-GTPase-activating protein SH3 domain-binding protein 1.

☆ The authors disclose no potential conflicts of interest.

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in vitro following the addition of quercetin (Chien et al., 2009), resveratrol (Sareen et al., 2007; Ma et al., 2007), EGCG (Kim et al., 2004), piceatannol (Liu and Chang, 2010) and similar products (Sergeev, 2004; Moon et al., 2013) 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 Williams, 2000). 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 (Berridge et al., 2000).

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 (Mattson and Chan, 2003; Joseph and Hajnóczky, 2007; Pinton et al., 2008). 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 (Pinton et al., 2008). In turn these small proteins induce the formation of the so-called apoptosome and subsequently the activation of a caspase cascade (Bao and Shi, 2007). We have shown that this “intrinsic” apoptotic pathway is one conduit to tumor cell death activated by several of the less toxic natural products(Van Ginkel et al., 2007; Sareen et al., 2007). 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 (Sareen et al., 2007). The depletion of ER calcium as a consequence of treatment with less toxic flavonoids and stilbenes also can instigate an ER stress response (Liu et al., 2014; Yeh et al., 2007; Park et al., 2007). Finally, the activity of several transcription factors, including NFκB and NFAT, is regulated by calcium (Brenner et al., 2007; Palkowitsch et al., 2011), affecting tumor development and progression (Aggarwal and Shishodia, 2006). 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 (El-Mowafy and Alkhalaf, 2003) and cAMP-PdE (Park et al., 2012) 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 (Van Ginkel et al., 2007), the family of calpains (Sareen et al., 2007) an ER stress response (Liu et al., 2014) and certain transcription factors (Aggarwal and Shishodia, 2006), 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% O_2/5% CO_2.

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 (Sareen et al., 2007). 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 μM 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× 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 s 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 5500 cells per well in a 96-well imaging plate one day prior to transfection. siRNAs were diluted to 0.25 μM 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× volume antibiotic free medium and plated onto the cells. Cells were transfected for 72 h followed by calcium imaging. Additional sets of wells were transfected for Western analysis of targeted proteins.

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