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Polymethoxylated flavones induce Ca^{2+}-mediated apoptosis in breast cancer cells

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Abstract

Flavonoids, polyphenolic phytochemicals which include flavones and isoflavones, are present in the common human diet. It has been suggested that these compounds may exert anticancer activity; however, the mechanisms involved remain unknown. We have recently shown (Sergeev, 2004, Biochem Biophys Res Commun 321: 462–467) that isoflavones can activate the novel apoptotic pathway mediated by cellular Ca^{2+}. Here, we report that polymethoxyflavones (PMFs) derived from sweet orange (*Citrus sinensis* L.) inhibit growth of human breast cancer cells via Ca^{2+}-dependent apoptotic mechanism. The treatment of MCF-7 breast cancer cells with 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-OH-HxMF) and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (3′-OH-TtMF) induced a sustained increase in concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) resulting from both depletion of the endoplasmic reticulum Ca^{2+} stores and Ca^{2+} influx from the extracellular space. This increase in $[Ca^{2+}]_i$ was associated with the activation of the Ca^{2+}-dependent apoptotic proteases, μ-calpain and caspase-12, as evaluated with the calpain and caspase-12 peptide substrates and antibodies to active (cleaved) forms of the enzymes. Corresponding non-hydroxylated PMFs, 3,5,6,7,8,3′,4′-heptamethoxyflavone (HpMF) and 5,6,7,3′,4′-pentamethoxyflavone (PtMF), were dramatically less active in inducing Ca^{2+}-mediated apoptosis. Our results strongly suggest that the cellular Ca^{2+} modulating activity of flavonoids underlies their apoptotic mechanism and that hydroxylation of PMFs is critical for their ability to induce an increase in $[Ca^{2+}]_i$ and, thus, activate Ca^{2+}-dependent apoptotic proteases.

Keywords: Intracellular calcium; Apoptosis; Calpain; Caspase-12; MCF-7 cells; Breast cancer; Flavonoids; Flavones; Methoxyflavones; Polymethoxyflavones; Sweet orange (*Citrus sinensis* L.)

Introduction

Flavonoids, a family of polyphenolic phytochemicals which include flavones and isoflavones, may exert anticancer activity, and diet high in such compounds tends to be associated with the reduced incidence of some cancers (Manthey et al., 2001; Cornwell et al., 2004). Flavonoids derived from sweet orange (*C. sinensis* L.) peel are intriguing anticancer agents because of their potential applications as chemotherapeutics and dietary supplements for cancer prevention. Orange peel is a rich source of flavonoids with polymethoxyflavones (PMFs) as major constituencies (Manthey and Guthrie, 2002). These compounds have been reported to express antiproliferative activity in cancer cells (Middleton et al., 2000; Manthey and Guthrie, 2002; Monasterio et al., 2004; Yanez et al., 2004; Zapata-Torres et al., 2004); however, the mechanism(s) involved has not been defined.

Induction of apoptotic cell death is a promising emerging strategy for prevention and treatment of breast cancer, because it may allow for differential elimination of cancer cells (Sergeev et al., 1998; Reed, 2003; Sergeev, 2005). Our findings (Sergeev et al., 1997, 2000; Sergeev and Rhoten, 1998; Sergeev and Norman, 2000; Sergeev, 2004a) indicate that regulation of apoptotic pathways in normal and cancer human mammary epithelial cells is different; therefore, characterization of the flavonoid's role in the fate of breast cancer cells may help in the rational search for the plant bioactive compounds that trigger apoptosis selectively in those cells.

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Cellular Ca^{2+} has been strongly implicated in induction of apoptosis and regulation of the apoptotic signaling pathways. We (Sergeev et al., 1998; Mathiasen et al., 2002; Sergeev, 2004a, 2005) and others (Berridge et al., 1998; Carafoli et al., 2001; Orrenius et al., 2003) have shown that an increase in concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) occurs in apoptosis. The critical characteristic of the apoptotic Ca^{2+} signal is a sustained increase in $[Ca^{2+}]_i$, reaching elevated, but not high, cytotoxic levels (Sergeev and Rhoten, 1998; Sergeev, 2004a, 2005). Recently, we described the novel, Ca^{2+}-mediated, calpain/caspase-12-dependent apoptotic pathway in breast cancer cells and showed that this pathway can be activated by the isoflavone genistein (Mathiasen et al., 2002; Sergeev and Norman, 2003; Sergeev, 2004a, 2004b, 2005).

The objective of this study was to investigate the ability of PMFs, derived from orange peel, to inhibit growth and induce apoptosis in human breast cancer cells. The hypothesis was that the growth inhibition of breast cancer cells by PMFs results from the activation of apoptosis. The findings obtained indicate that PMFs induce a sustained increase in $[Ca^{2+}]_i$, activate Ca^{2+}-dependent μ-calpain and caspase-12, and induce apoptosis in breast cancer cells and that hydroxylations of PMFs are required to confer their proapoptotic activity.

Materials and methods

General separation procedures from crude sweet orange peel extract (OPE). The crude OPE mixture containing 40% PMFs obtained from Florida Flavors, Inc. (10 g) was dissolved in a mixture of methylene chloride (2 mL) and hexanes (2 mL) and loaded onto a 120 g pre-conditioned silica gel flash column. The gradient was started with 10% ethyl acetate and 90% hexanes and went to 40% ethyl acetate and 60% hexanes within 35 min. Then the isocratic mobile phase (40% ethyl acetate–60% hexanes) was applied for another 15 min (total run of 50 min). The fractions that had UV absorbance at 254 nm were analyzed by LC-ESI-MS, and the fractions were combined into several groups according to their molecular weight obtained from LC/MS analysis. Further separation led to isolation of pure 3,5,6,7,8,3′,4′-heptamethoxyflavone (HpMF) and 5,6,7,3′,4′-pentamethoxyflavone (PtMF).

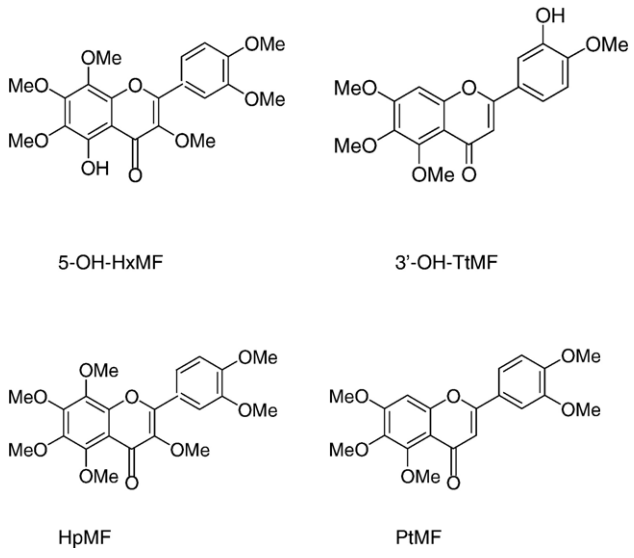

Fig. 1. Structures of polymethoxyflavones.

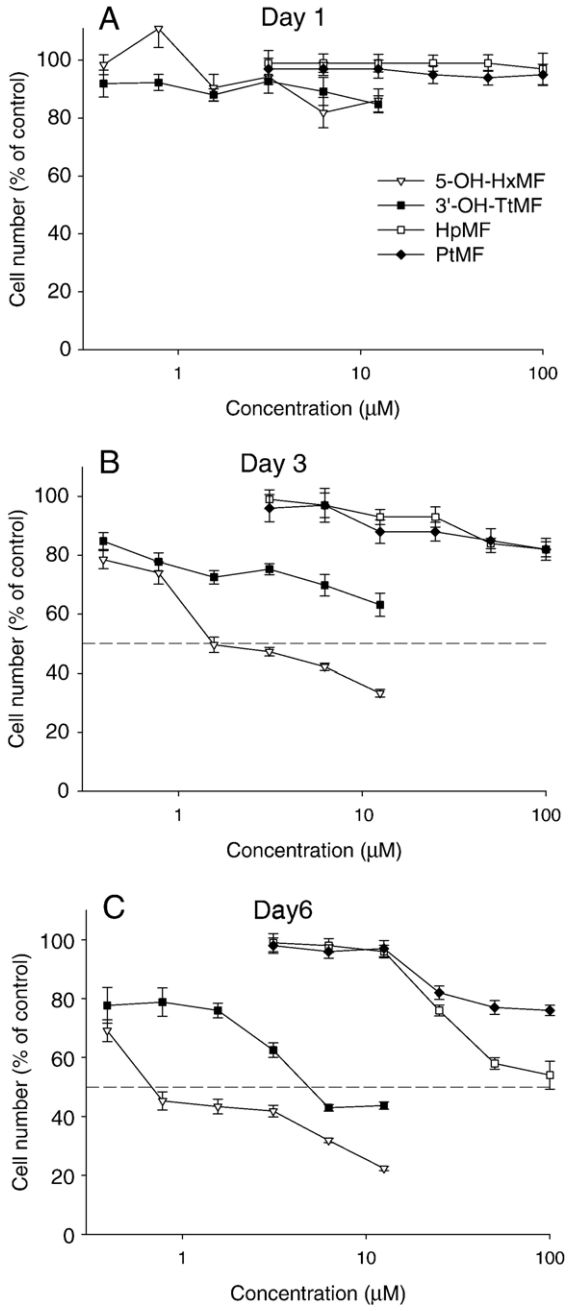

Fig. 2. Growth inhibitory effects of PMFs in MCF-7 cells. Cell numbers, presented as percent of control, were determined as described in the Methods section. Here and in Figs. 3–5, cells were treated with PMFs or vehicle for 1 (A), 3 (B) or 6 (C) days. Results are means±SE of triplicate determinations of two independent experiments. *X* axis is log (common). Dash lines in Fig. 2B, C indicate a 50% level.

Separation procedures of hydroxylated PMFs. The fractions that contain hydroxylated PMFs characterized by LC-ESI-MS, were concentrated and then dissolved in acetonitrile and water. The dissolved solution was loaded onto a C_{18} reverse phase HPLC system. A gradient method was used from 25%

acetonitrile–75% water to 60% acetonitrile–40% water in 25 min with a flow rate of 20 mL/min. The fractions were analyzed by LC-ESI-MS. Both the pure compounds and mixtures were collected. The pure fractions by LC-MS were combined and concentrated or lyophilized to dryness and the dried compounds were analyzed by MS, UV and NMR. The mixtures were concentrated on rotovapor and dissolved in minimum amount of methylene chloride. The solution was then loaded onto the HPLC system equipped with the Welk-O 1 (R, R) Regis column (450) gram. The monitoring UV absorbance was set at 280 nm. The fractions were collected and concentrated respectively. MS, UV and NMR were taken for these fractions. 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-OH-HxMF) was obtained from these fractions. 3′-hydroxy-5,6,7,4′-tetramethoxyflavone was purchased from Alfa Aesar (Ward Hill, MA).

Cell culture. The human breast carcinoma cell line MCF-7 (ATTC) was used in this study. MCF-7 cells were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were treated with PMFs (at nine concentrations ranging from 0.39 μM to 100 μM) or vehicle, dimethylsulfoxide (DMSO; 0.1%), for 1, 3 or 6 days. Stock solutions of PMFs (200 mM) were prepared in DMSO. 5-OH-HxMF and 3′-OH-TtMF were not used at concentrations exceeding 25 μM due to their poor solubility in the cell culture medium at higher concentrations.

The treatments of cells started 24 h after seeding. Cell growth was evaluated by counting the cell numbers in hemacytometer and measuring the cellular total nucleic acid content with the CyQUANT Cell Proliferation Assay Kit (Molecular Probes).

Apoptosis. Apoptosis was evaluated by the plasma membrane and nuclear changes. Annexin V assay (Alexa Fluor 488 Annexin V Assay Kit; Molecular Probes) was used for the detection of the apoptotic plasma membrane (loss of membrane asymmetry due to phosphatidylserine translocation) (Sergeev, 2004a). Fluorescence (485 nm excitation, 530 nm emission) of the Annexin V-labeled cells grown in 96-well plates was measured in the FLx800 plate reader with KC software (BioTek) and expressed in fluorescence units per 1×10^3 cells. Propidium iodide uptake was used to evaluate non-apoptotic and apoptotic cell death (the dye permeates plasma membrane and stain nucleic acids of both necrotic and the late apoptotic cells)

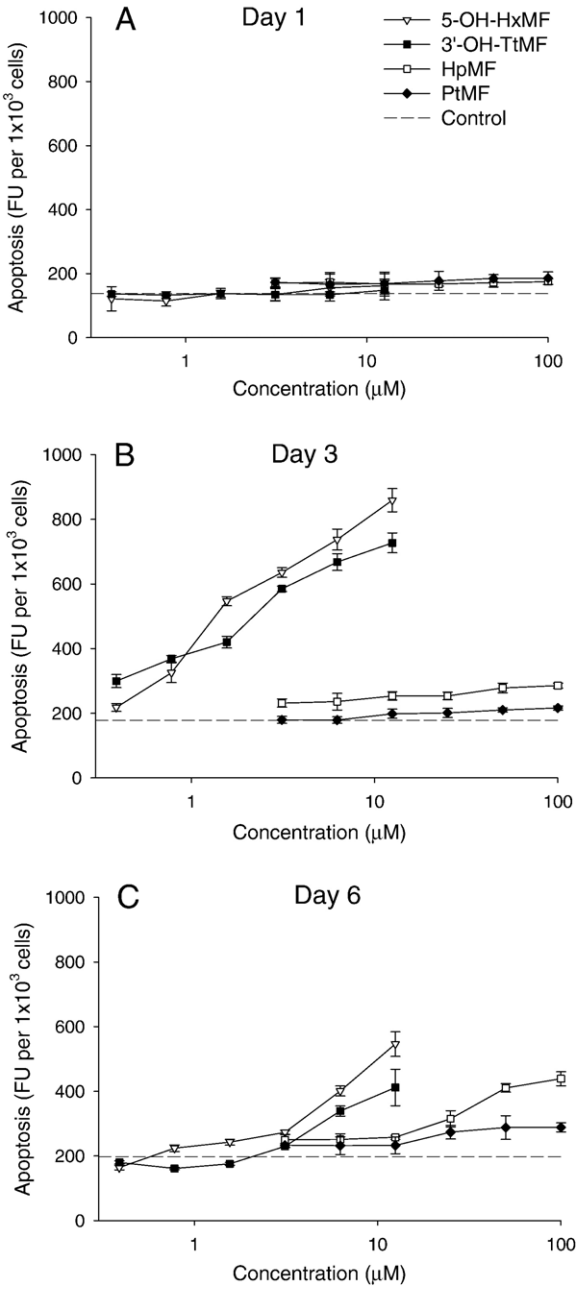

Fig. 3. Proapoptotic effect of PMFs in MCF-7 cells. Apoptosis was measured with Annexin V, as described in the Methods section. Here and in Figs. 4 and 5, results are presented as fluorescence intensity units (FU) per 1×10^3 cells.

Table 1
Effective concentrations of PMFs in MCF-7 breast cancer cells

Compound	Inhibition of cell proliferation (IC_{50}, μM)	Induction of cell apoptosis (EC_{min}, μM)	Induction of cell death (EC_{min}, μM)	Increase in $[Ca^{2+}]_i$ (EC_{min}, μM)
5-OH-HxMF	2.50	1.56	3.125	1.56
3′-OH-TtMF	10.5	3.125	6.25	3.125
HpMF	>50	50	>50	50
PtMF	>50	>50	>50	>50

IC_{50}, the concentration inhibiting cell growth by 50%, is presented as the average of the day 3 and day 6 determinations. The statistically significant minimal effective concentrations, EC_{min}, for induction of cell death, apoptosis, and increase in $[Ca^{2+}]_i$ are also indicated. Effects of PMFs on proliferation, apoptosis, death, and $[Ca^{2+}]_i$ levels in MCF-7 cells were evaluated as described in the Methods section.

(Sergeev, 2004a). Fluorescence (530 nm excitation, 620 nm emission) of the propidium iodide-labeled cells was also measured in the FLx800 reader. Additionally, Hoechst 33342 and Alexa Fluor 488 Annexin V were employed to visualize apoptotic nuclei (nuclear fragmentation) and the apoptotic plasma membrane, respectively. Fluorescence microscopy of Hoechst 33342- and Annexin V-labeled cells was performed as described below for the cellular Ca^{2+} and immunofluorescence imaging.

Intracellular calcium. Concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured with Ca^{2+} indicators fura-2 and fluo-3, as described in detail previously (Sergeev and Rhoten, 1995, 1998;

Sergeev, 2004a). For $[Ca^{2+}]_i$ measurements with fluo-3, cells grown in the 96-well, black-wall plates were loaded with 2 μM of fluo-3/AM (Molecular Probes) in Dulbecco's PBS (D-PBS) supplemented with 0.1% DMSO for 40 min at 37 °C. Fluorescence (485 nm excitation, 530 nm emission) was measured in the FLx800 plate reader, as described above. For $[Ca^{2+}]_i$ measurements with fura-2, cells grown on coverslips were loaded with 1 μM of fura-2/AM (Molecular Probes) in D-PBS supplemented with 0.1% DMSO and 0.01% Pluronic F-127 for 40 min at 37 °C. The dynamics of intracellular Ca^{2+} was assessed with cells in the microincubation chamber (37.0±0.2 °C) on a Nikon Eclipse TE-300 inverted microscope equipped for epifluorescence, ratiometric, digital imaging. The images were captured using SuperFluor 40X 1.3 NA oil-immersion objective (Nikon) and CoolSnapFX digital CCD camera (Photometrics), ratioed (340/380 nm excitation, 510 nm emission) on a pixel-by-pixel basis, and stored for analysis. Image analysis was performed using MetaFluor 6.3 software (Molecular Devices/Universal Imaging).

To evaluate Ca^{2+} influx from the extracellular space (Sergeev and Rhoten, 1995, 1998), the Mn^{2+} entry rate, as a reporter of Ca^{2+} influx, was measured. The images were recorded at an excitation of 360 nm (the fura-2 isosbestic point) and 2 mM of extracellular Mn^{2+}. The rates of Ca^{2+} entry were estimated from the slope of the linear portion of curves of the fura-2 fluorescence quench by Mn^{2+}. To evaluate Ca^{2+} release from the intracellular stores (Rhoten and Sergeev, 1994; Sergeev, 2004a), cells grown on coverslips were placed in D-PBS, and the mobilizer of the endoplasmic reticulum Ca^{2+} stores, thapsigargin (1 μM), was added after recording the basal $[Ca^{2+}]_i$. The peak values of the $[Ca^{2+}]_i$ increase, indicating filling levels of the Ca^{2+} stores, were measured with fura-2.

Calpain and caspase-12. Calpain and caspase-12 activities were measured with the cell-permeable fluorogenic peptide substrates t-Boc-Leu-Met-CMAC (50 μM; CMAC, 7-amino-4-chloromethyl coumarin; Molecular Probes) (Roser and Gores, 2000) and Ala-Thr-Ala-Asp-AFC (50 μM; AFC, 7-amino-4-trifluoromethyl coumarin; Caspase-12 Fluorometric Assay Kit, BioVision), respectively. Polyclonal antibodies directed against caspase-12 (BioVision) and monoclonal antibodies directed against the calpain small (cleaved) subunit (Chemicon) were used to evaluate the activation of these proteases. We (Sergeev and Norman, 2003; Sergeev, 2004a, b) and others (Zhu et al., 2001) have shown previously that the Ca^{2+}-activated calpain and the Ca^{2+}/calpain-activated caspase-12 are expressed in the apoptotic MCF-7 cells.

For immunofluorescence labeling, the fixed and permeabilized cell preparations were pre-incubated with non-specific serum for 20 min, incubated for 1 h at 37 °C or overnight at 4 °C with primary antibodies and 1 h at room temperature with secondary antibodies (Alexa Fluor-488 signal-amplification mouse antibodies (Molecular Probes) and FITC- or Texas Red-conjugated anti-rabbit and anti-mouse IgG (Molecular Probes). Fluorescence microscopy was carried out as described above for Ca^{2+} imaging. Image analysis and measurement of fluorescence intensity were performed using MetaMorph 6.3 software

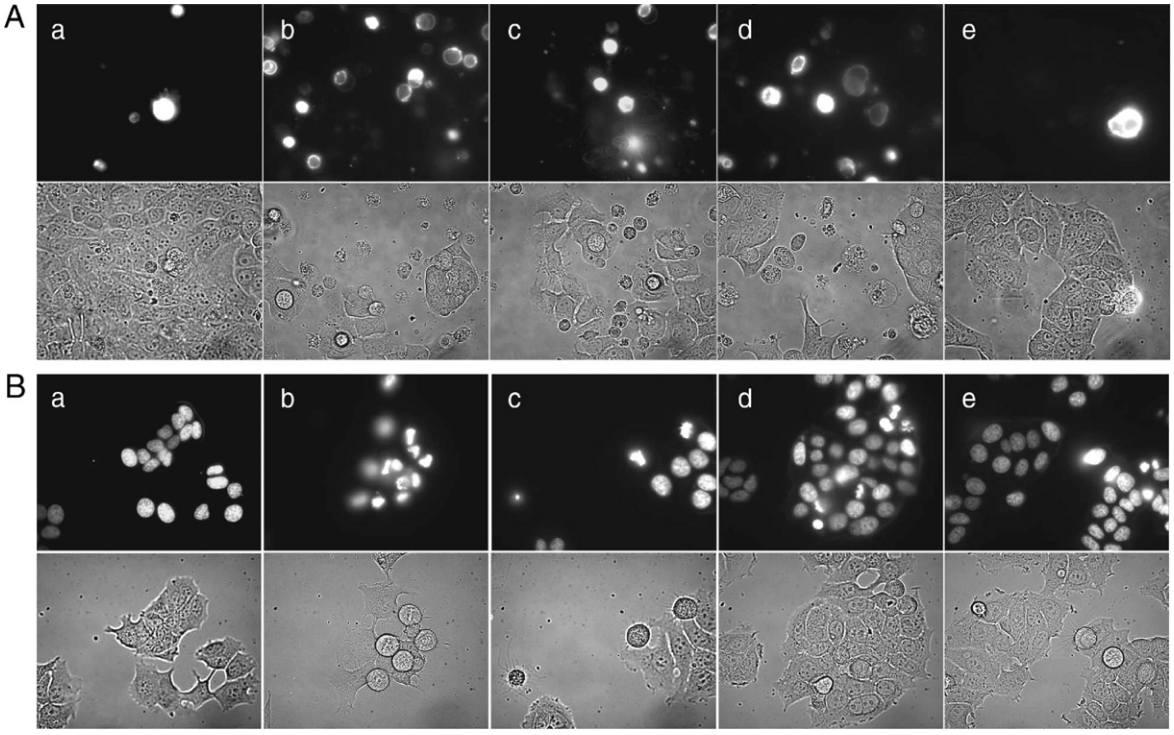

Fig. 4. The plasma membrane asymmetry and nuclear fragmentation in PMF-treated MCF-7 cells. (A), Annexin V-labeled cells; (B), Hoesht 33342-labeled cells. Upper rows, fluorescence images; lower rows, phase-contrast images; a, control; b, 5-OH-HxMF; c, 3′-OH-TtMF; d, HpMF; e, PtMF. The cells were treated with PMFs for 6 (A) or 3 (B) days at concentrations indicated in the legend to Fig. 7. Note the labeling of, and nuclear fragmentation/DNA condensation in, the apoptotic (round) cells. See Methods and Results sections for additional explanations.

(Molecular Devices/Universal Imaging), as described previously (Sergeev et al., 1996).

Results

Compounds. Structures of pure 5-OH-HxMF, 3′-OH-TtMF, HpMF, and PtMF are shown in Fig. 1.

Cell growth and apoptosis. 5-OH-HxMF, 3′-OH-TtMF, HpMF, and PtMF inhibited proliferation of MCF-7 breast cancer cells in a dose- and time-dependent fashion, as evaluated by counting the cell numbers (Fig. 2) and determining an increase in the cellular total nucleic acid content (not shown; similar results as with the cell counts). The IC_{50} values for inhibition of cell proliferation are presented in Table 1. Relative efficacy of the tested compounds for the antiproliferative activity ranked as follows: 5-OH-HxMF>3′-OH-TtMF>> HpMF>PtMF. It is important to note that HpMF and PtMF exhibited low antiproliferative activity in comparison with 5-OH-HxMF and 3′-OH-TtMF. The tested compounds did not exert the cytotoxic effect, as evident by no changes in the numbers of viable cells after a 1-day treatment (see Fig. 2).

5-OH-HxMF and 3′-OH-TtMF induced apoptosis in MCF-7 breast cancer cells at day 3 and 6 of treatment in a dose-dependent manner (as measured with the fluorescent probe

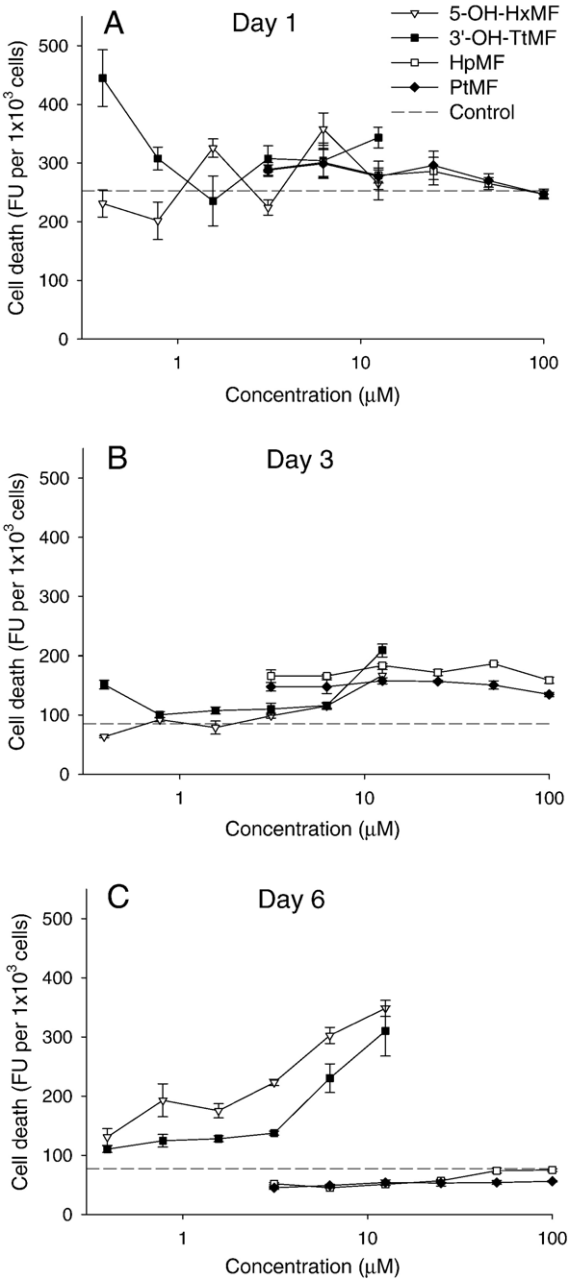

Fig. 5. Death induced by PMFs in MCF-7 cells. Cell death was evaluated with propidium iodide, as described in the Methods section. The elevated fluorescence intensity levels at day 1, as compared with day 3 and 6, indicate higher permeability of the plasma membrane to propidium iodide of cells recovering after trypsinization at day 1.

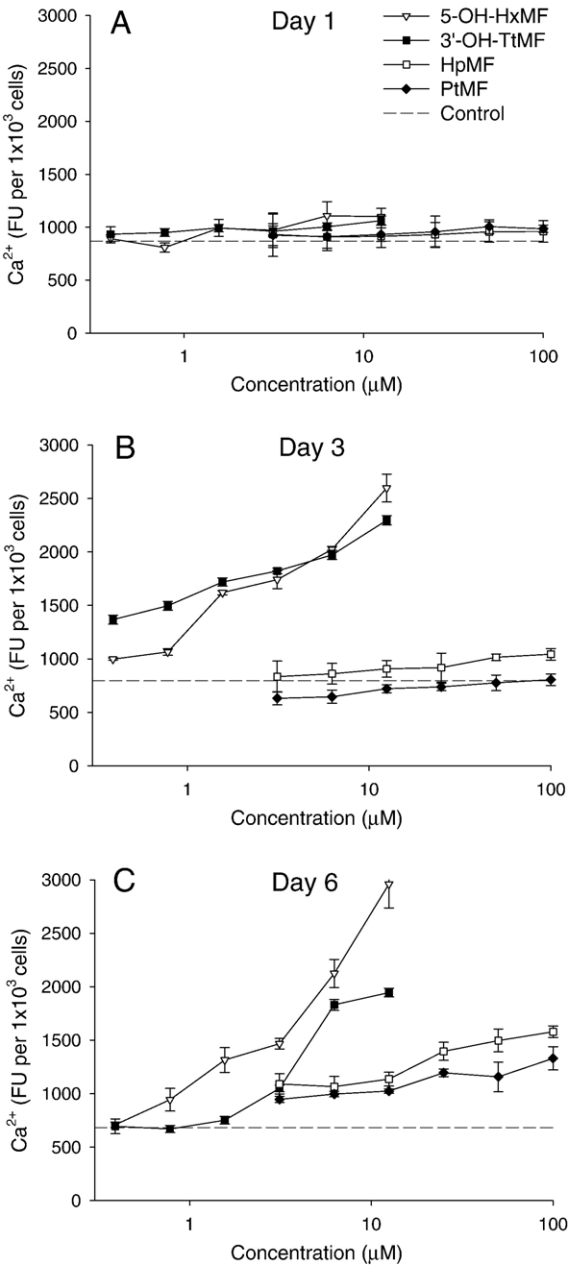

Fig. 6. Effect of PMFs on intracellular Ca^{2+} levels in MCF-7 cells. $[Ca^{2+}]_i$ was measured with fluo-3/AM, as described in the Methods section.

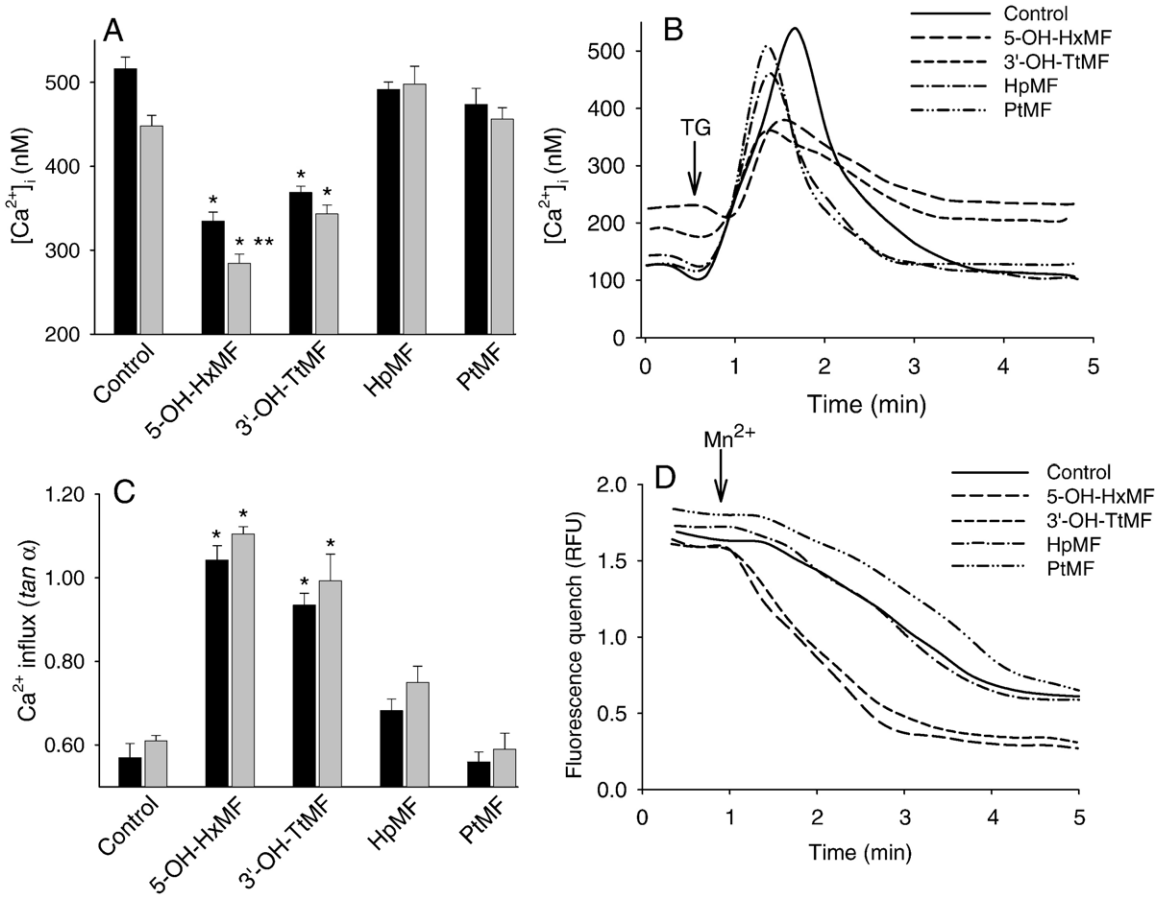

Fig. 7. Effects of PMFs on Ca^{2+} influx and Ca^{2+} mobilization in MCF-7 cells. $[Ca^{2+}]_i$ and the Ca^{2+} entry rate were measured with fura-2 and employing the fura-2 fluorescence quench, respectively, as described in Methods. The Ca^{2+} mobilization responses (A, B) are shown as the maximum $[Ca^{2+}]_i$ rises after addition of thapsigargin. The Ca^{2+} entry rates (C, D) are presented as tangents of the linear portions of the fura-2 quench curves. Here and in Fig. 8, data are presented as means ± SE; (*), $p<0.05$, as compared with the corresponding control group; (**), $p<0.05$, as compared with the day 3 treatment group. The following effective concentrations of the tested PMFs were used: 5-OH-HxMF, 6.25 μM; 3′-OH-TtMF, 12.5 μM; HpMF, 25 μM; and PtMF, 25 μM. The cells were treated with PMFs at these concentrations for 3 (black bars) or 6 (gray bars) days. Panels D and B, representative traces of the single cell recordings of the Ca^{2+} influx and Ca^{2+} mobilization, respectively. RFU, relative fluorescence units; Mn^{2+} or thapsigargin (TG) were added after recording the basal level of fluorescence or basal $[Ca^{2+}]_i$ for 30-60 s.

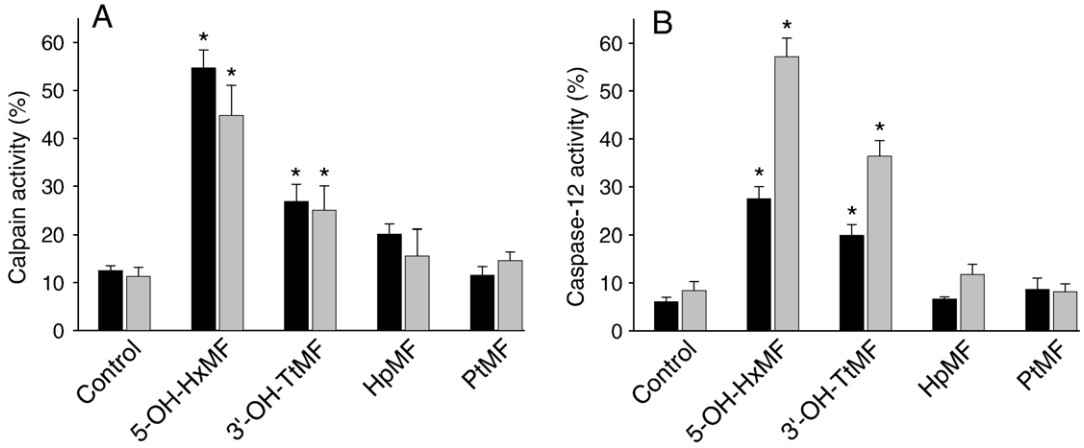

Fig. 8. Effects of PMFs on calpain and caspase-12 activity in MCF-7 cells. Calpain (A) and caspase-12 (B) activities were measured with the fluorogenic peptide substrates at day 3 (black bars) or 6 (gray bars) and expressed as percentage of the fluorescently labeled cells (defined as cells with fluorescence intensity at least 2.5-fold above the background cell fluorescence). See legend to Fig. 7 and Methods and Results sections for additional explanations.

Alexa Fluor 488 Annexin V; Fig. 3). The effective concentrations were similar to those for antiproliferative activity (see Table 1). HpMF demonstrated low proapototic activity at higher concentrations (25–100 μM) at day 6 of treatment. PtMF showed only a trend in inducing apoptosis at day 6 of treatment (50–100 μM). Morphological criteria (nuclear fragmentation/

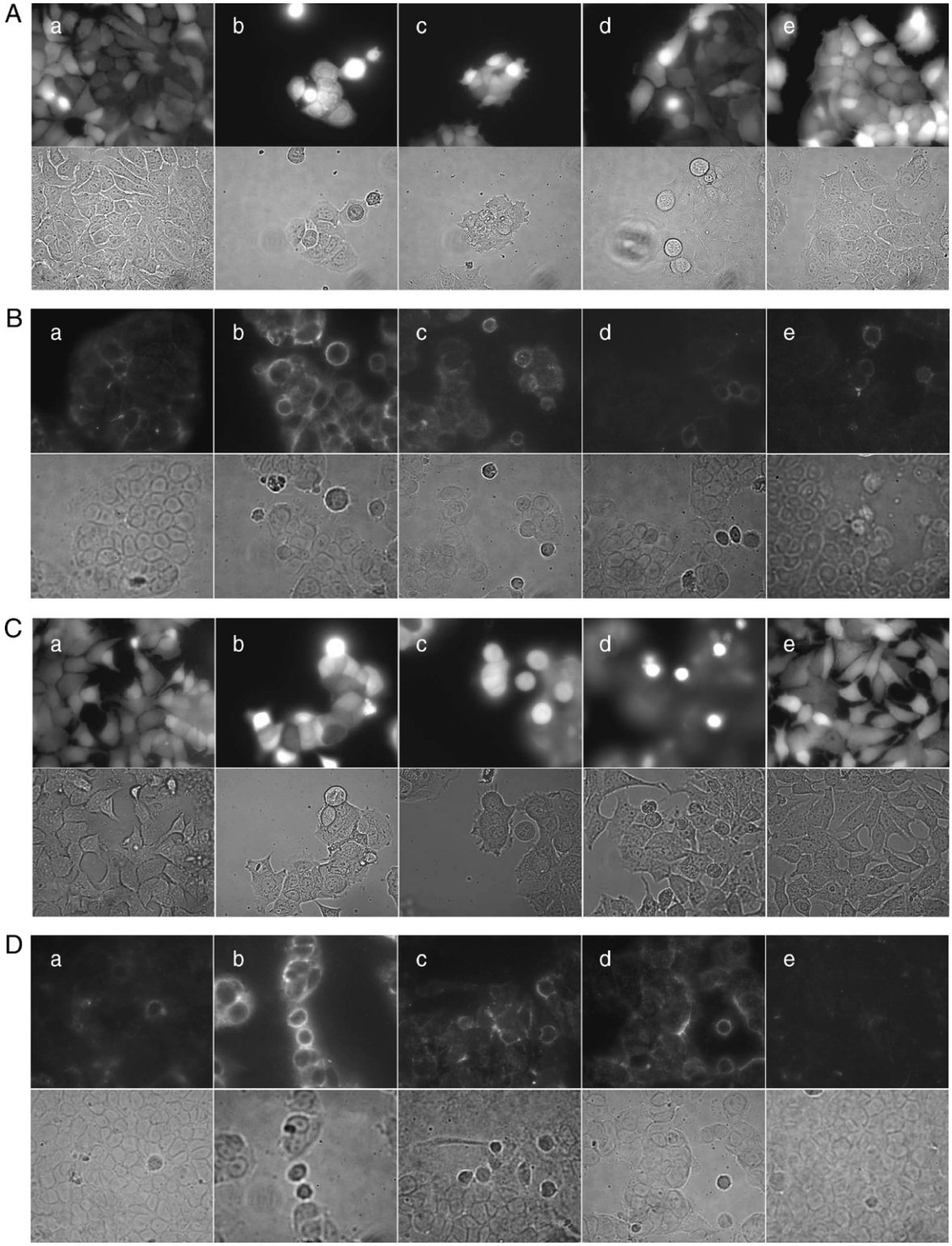

Fig. 9. Calpain and caspase-12 activation in PMF-treated MCF-7 cells. (A), the cleaved calpain substrate; (B), the calpain small subunit; (C), the cleaved caspase-12 substrate; (D), the caspase-12 protein. Cells were treated with PMFs for 3 (calpain) or 6 (caspase-12) days at concentrations indicated at the legend to Fig. 7. Upper rows, fluorescence images; lower rows, phase-contrast images; a, control; b, 5-OH-HxMF; c, 3′-OH-TtMF; d, HpMF; e, PtMF. Note calpain activation/cleavage and caspase-12 expression/activation in the apoptotic (round) cells. See Methods and Results sections for additional explanations.

condensation) confirmed apoptosis in the cells treated with 5-OH-HxMF, 3′-OH-TtMF, and HpMF (Fig. 4).

5-OH-HxMF and 3-OH-TtMF induced death of MCF-7 breast cancer cells (as evaluated with the propidium iodide; Fig. 5, also see Table 1). Cell death was evident at day 6 of treatment; apparently, it resulted from an increasing number of the late stage apoptotic cells. HpMF and PtMF were not effective in inducing non-apoptotic cell death.

Intrtacellular Ca^{2+}. 5-OH-HxMF and 3-OH-TtMF induced an increase in the basal level of $[Ca^{2+}]_i$ in breast cancer cells at day 3 and 6 of treatment in a dose-dependent fashion (as evaluated with fluo-3; Fig. 6). Effective concentrations were similar to those for the antiproliferative and proapoptotic activities (see Table 1). HpMF and PtMF showed only a trend in increasing intracellular Ca^{2+} levels at higher concentrations (50–100 μM) at day 6 of treatment.

To investigate the mechanism of a sustained increase in $[Ca^{2+}]_i$ after treatment with PMFs, the rate of the background Ca^{2+} influx from the extracellular space and the magnitude of Ca^{2+} release from the endoplasmic reticulum stores with thapsigargin were measured (Sergeev and Rhoten, 1995, 1998). The Ca^{2+} mobilization response was significantly (1.5- and 1.35-fold, respectively) decreased in cells treated with 5-OH-HxMF and 3′-OH-TtMF (Fig. 7A, B), indicating that the tested compounds did deplete intracellular Ca^{2+} stores. The smaller Ca^{2+} mobilization response of 5-OH-HxMF at day 6 of treatment, as compared with the response at day 3, implies that the Ca^{2+} stores were chronically depleted at the later point. HpMF and PtMF did not significantly affect the filling of the endoplasmic reticulum Ca^{2+} stores.

5-OH-HxMF and 3′-OH-TtMF, but not HpMF and PtMF markedly (1.8- and 1.6-fold, respectively) increased Ca^{2+} influx (Fig. 7C, D), as evaluated by the Ca^{2+} entry rates via the low-conductance Ca^{2+} channels. It is noteworthy that the return of $[Ca^{2+}]_i$ to basal levels after treatment with thapsigargin was slower in cells treated with 5-OH-HxMF and 3′-OH-TtMF than in controls and cells treated with HpMF and PtMF (see Fig. 7B). The prolonged "$[Ca^{2+}]_i$ decreasing" phase of the response to thapsigargin in Ca^{2+}-containing solutions indicates Ca^{2+} entry (Sergeev and Rhoten, 1995); therefore, the mentioned above observation is consistent with the notion that 5-OH-HxMF and 3′-OH-TtMF increase Ca^{2+} influx. Taken together, these findings indicate that an elevated $[Ca^{2+}]_i$ in the cells treated with 5-OH-HxMF and 3′-OH-TtMF results from both Ca^{2+} influx from the extracellular space and depletion of the intracellular Ca^{2+} stores.

Calpain and caspase-12. A sustained increase in $[Ca^{2+}]_i$ in MCF-7 cells treated with 5-OH-HxMF and 3′-OH-TtMF was accompanied by the activation of the Ca^{2+}-dependent apoptotic proteases, μ-calpain and caspase-12. The calpain activation was demonstrated by cleavage of the fluorogenic peptide calpain substrate t-Boc-Leu-Met (Figs. 8A and 9A) and the calpain protein (i.e., presence in cells of the calpain small subunit) (Fig. 9B). Caspase-12 activation in cells treated with 5-OH-HxMF and 3-OH-TtMF was shown with the peptide substrate ATAD (Figs. 8B and 9C) and demonstrated with the monoclonal antibodies recognizing truncated caspase-12 (Fig. 9D). No significant calpain and caspase-12 activation was detected in cells treated with HpMF and PtMF, although HpMF demonstrated a trend in increasing the number of cells with activated calpain at day 3 of treatment (see Fig. 8A). Noteworthy, caspase-12 was not expressed/activated in the non-apoptotic MCF-7 cells (see Fig. 9D; also, Sergeev and Norman, 2000). Taken together, these results imply that 5-OH-HxMF and 3′-OH-TtMF induce Ca^{2+}-dependent activation of calpain and caspase-12.

Discussion

The results presented in this paper provide evidence for the antiproliferative and proapoptotic activity of PMFs in breast cancer cells and support the hypothesis that the PMF-induced sustained increase in $[Ca^{2+}]_i$ is associated with induction of apoptosis in these cells. Our findings also strongly imply that induction of apoptosis with PMFs requires activation of the Ca^{2+}-dependent μ-calpain and the Ca^{2+}/calpain-dependent caspase-12 and that hydroxylations of PMFs are critical for enhancing their proapoptotic activity. Importantly, PMFs exhibited proapoptotic activity in breast cancer cells at physiologically relevant concentrations, which can be achieved by consuming high amounts of orange-derived products or PMF supplements (Yu, 2004).

An increase of intracellular Ca^{2+} induces apoptosis in various cell models (Berridge et al., 1998; Mathiasen et al., 2002; Orrenius et al., 2003; Sergeev, 2004a). Here, we report that the PMF-induced sustained increase in $[Ca^{2+}]_i$ triggers apoptosis in human breast carcinoma cells. The $[Ca^{2+}]_i$ increase was due to both Ca^{2+} influx from the extracellular space and Ca^{2+} mobilization from the endoplasmic reticulum stores. The mechanism of generating the Ca^{2+} response with PMFs may include their interactions with membrane receptors coupled to the low-conductance Ca^{2+} channels. For example, we have shown previously (Sergeev and Rhoten, 1998; Sergeev, 2004b; Sergeev, 2005) that the vitamin D hormone, $1,25(OH)_2$-vitamin D_3, and isoflavones (genistein) may interact with their appropriate membrane receptors (vitamin D receptor or estrogen receptor) to evoke a sustained increase in $[Ca^{2+}]_i$ associated with induction of apoptosis. PMFs may directly or indirectly influence the Ca^{2+} regulatory responses of these membrane receptors, e.g., affecting their membrane environment (particularly, hydroxylated PMFs) or regulating receptors at the genomic level. Hydroxylation of flavones appears to be crucial for conferring their estrogenic activity (Zand et al., 2000), while position and number of methoxy groups may be important for modulating this activity.

The PMF-induced increase in $[Ca^{2+}]_i$ resulted in the activation of apoptotic proteases, the Ca^{2+}-dependent μ-calpain and the Ca^{2+}/calpain-dependent caspase-12. These data are in agreement with our (Mathiasen et al., 2002; Sergeev and Norman, 2003; Sergeev, 2004a, b) and others (Nakagawa et al., 2000) findings indicating that an increase in $[Ca^{2+}]_i$ is necessary for calpain and caspase-12 activation and that caspase-12 requires calpain for its cleavage/activation. It is important to note that the caspase-12 gene expresses polymorphism in humans (with significant expression in African-Americans) (Kalai et al., 2003; Ho and Hawkins, 2005), and MCF-7 cells express the inducible Ca^{2+}/calpain-activated caspase-12-like

protein, which is recognized with antibodies against rodent caspase-12 (Zhu et al., 2001; Sergeev, 2004b).

Collectively, results presented here further support the hypothesis that a sustained increase in $[Ca^{2+}]_i$ induces apoptosis and that such an increase in cellular Ca^{2+} leads to activation of Ca^{2+}-dependent apoptotic proteases. The PMF-induced Ca^{2+}-mediated apoptotic mechanism can be exploited for induction of cell death in certain diseases. For example, differential regulation of cellular Ca^{2+} in normal vs. cancer human mammary epithelial cells may allow the selective induction of Ca^{2+}-mediated apoptotic cell death in breast cancer (Sergeev, 2004a, 2005), and structural differences between hydroxylated and non-hydroxylated PMFs could be exploited for selecting or designing agents with proapoptotic activity in breast cancer cells.

In conclusion, we have demonstrated that the apoptotic pathway in breast carcinoma cells treated with PMFs includes the following events: sustained increase in $[Ca^{2+}]_i \rightarrow$ μ-calpain activation → caspase-12 activation → apoptosis.

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