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Polymethoxyflavones Activate Ca^{2+}-Dependent Apoptotic Targets in Adipocytes

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Induction of apoptosis is an emerging strategy for the prevention and treatment of obesity because removal of adipocytes via apoptosis will result in reducing body fat and may help to maintain a long-lasting weight loss. Our previous studies have shown that a sustained increase in intracellular Ca^{2+} triggers apoptosis in various cell types via activation of Ca^{2+}-dependent proteases and that the apoptosis-inducing effect of polymethoxyflavones (PMFs) in cancer cells is mediated through Ca^{2+} signaling. This paper reports that PMFs induce apoptosis in mature mouse 3T3-L1 adipocytes via activation of Ca^{2+}-dependent calpain and Ca^{2+}/calpain-dependent caspase-12. Treatment of adipocytes with PMFs evoked, in a concentration- and time-dependent fashion, sustained increase in the basal level of intracellular Ca^{2+}. The increase in Ca^{2+} was associated with induction of apoptosis and activation of μ-calpain and caspase-12. Apoptosis-inducing activity of hydroxylated PMFs was significantly higher than that of the corresponding nonhydroxylated compounds. These results demonstrate that the apoptotic molecular targets activated by PMFs in adipocytes are Ca^{2+}-dependent calpain and caspase-12. The findings obtained provide rationale for evaluating the role of PMFs in the prevention and treatment of obesity.

KEYWORDS: Polymethoxyflavones; intracellular Ca^{2+}; apoptosis; calpain; caspase-12; adipocytes; 3T3-L1 cells; obesity

INTRODUCTION

Obesity remains a serious public health issue with a cohort of new studies providing details on the magnitude of the problem and its associated health complications (*1*). A combination of dietary, behavioral, and therapeutic approaches is required for effective prevention and treatment of obesity, and even when successful, weight regain occurs in a majority of patients. The discovery of adipogenic precursors (*2*) and the possibility of targeting adipocytes for apoptotic cell death as a weight loss strategy (*3*) raise the possibility of an alternative approach to longer term weight management. Decreasing adiposity through induction of adipocyte apoptosis may result in long-lasting maintenance of weight loss, in contrast to that which occurs after caloric restriction or bariatric surgery. There is a growing body of evidence that phytochemicals can inhibit adipogenesis and induce apoptosis in the fat tissue (*4*−*7*). However, a better understanding of the cellular and molecular events leading to adipocyte apoptosis is necessary to successfully employ this approach.

The increase in the adipose tissue mass is the result of both hypertrophy, an increase in adipocyte size, and hyperplasia, an increase in adipocyte number (*8*−*10*). Therefore, weight loss can be caused by a decrease not only in adipocyte size but also in adipocyte number (i.e., by stimulating apoptosis). An increase in the rate of adipocyte apoptosis will prevent excessive accumulation of adipose tissue and may result in a significant loss of adipose tissue mass over time. It has been shown recently that within the stable population of adipocytes in adults approximately 10% of fat cells are renewed annually (*11*), which may approximate a normal rate of apoptosis in this tissue.

Polymethoxyflavones (PMFs) are intriguing bioactive plant compounds. They exist almost exclusively in the *Citrus* genus, particularly in the peel of sweet oranges (*C. sinensis*) and mandarin oranges (*C. reticulate*). PMFs have a broad spectrum of biological activity including the expression of apoptosis-inducing activity in various cell types (*12*−*15*). Monohydroxy-PMFs and dihydroxy-PMFs, which are minor components of citrus peel or metabolites of PMFs, appear to have stronger antitumor activity relative to their counterpart PMFs (*16*).

A critical regulator of the apoptotic signaling pathways is cellular Ca^{2+}. We have shown that the necessary characteristic of the apoptotic Ca^{2+} signal is a sustained increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$), reaching elevated, but not cytotoxic, levels (*17*−*20*). Such a $[Ca^{2+}]_i$ increase is associated with activation of apoptotic proteases, Ca^{2+}-dependent calpains, and several caspases (*19*, *21*−*25*). We have also shown previously

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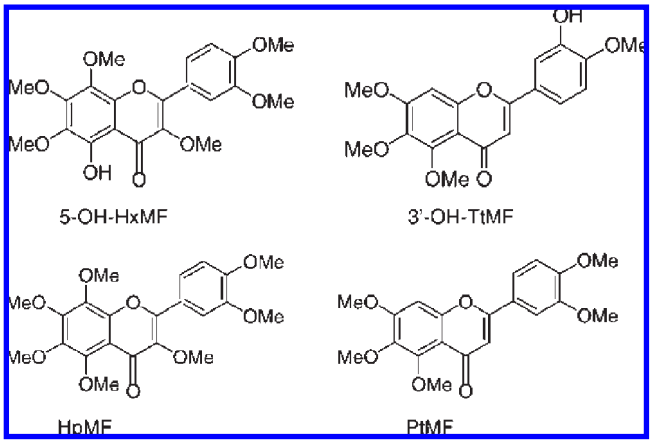

Figure 1. Chemical structures of PMFs.

that PMFs activate the Ca^{2+}-mediated, calpain/caspase-dependent apoptotic pathway in human breast cancer cells, namely, a sustained increase in intracellular Ca^{2+} → μ-calpain activation → caspase-12 activation → apoptosis (*12*, *13*).

The study presented here was undertaken to determine whether PMFs can induce Ca^{2+}-mediated apoptosis in mature adipocytes. We hypothesized that PMFs would trigger an increase in $[Ca^{2+}]_i$ followed by activation of Ca^{2+}-dependent apoptotic molecular targets in these cells. The results obtained demonstrate that PMFs increase basal levels of intracellular Ca^{2+}, activate apoptotic proteases, Ca^{2+}-dependent calpain, and Ca^{2+}/calpain-dependent caspase-12, and induce apoptosis in mature adipocytes.

MATERIALS AND METHODS

Polymethoxyflavones. 5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-OH-HxMF), 3,5,6,7,8,3′,4′-heptamethoxyflavone (HpMF), and 5,6,7,3′,4′-pentamethoxyflavone (PtMF) were isolated from crude sweet orange extract obtained from Florida Flavors, Inc., as described in our previous publication (*12*). 3′-Hydroxy-5,6,7,4′-tetramethoxyflavone (3′-OH-TtMF) was purchased from Alfa Aesar (Ward Hill, MA). The chemical structures of PMFs used in this study are presented in **Figure 1**.

Cell Culture. The established, well-characterized 3T3-L1 preadipocyte cell line (ATCC) was used in these experiments. Preadipocyte maturation was induced with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin (*26*). 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% adult bovine serum and 1% penicillin−streptomycin (10000 U/mL penicillin and 10000 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 10% CO_2 in air. To induce differentiation, 2-day-postconfluent preadipocytes were stimulated for 48 h with 0.5 mM IBMX, 0.5 μM dexamethasone, and 10 μg/mL insulin (MDI) added to DMEM/10% fetal bovine serum (FBS) culture medium. After 2 days of stimulation, the MDI medium was replaced with DMEM/10% FBS. Treatment of mature adipocytes with PMFs or vehicle, dimethyl sulfoxide (DMSO, 0.1%), started at this point (day 0) and continued for 1, 3, or 6 days. Cells were treated with PMFs (at six concentrations ranging from 3.125 to 100 μM) or vehicle for 1, 3, or 6 days. Stock solutions of PMFs (200 mM) were prepared in DMSO.

Intracellular Ca^{2+}. For $[Ca^{2+}]_i$ measurements (*12*, *27*, *28*), cells grown in the 96-well, black-wall plates were loaded with 2 μM 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 with KC software (BioTek) and expressed in relative fluorescence units (RFUs) per well.

Apoptosis. Apoptosis was evaluated by plasma membrane changes on the basis of the Annexin V assay (Alexa Fluor 488 Annexin V Assay Kit; Molecular Probes) for monitoring the apoptotic plasma membrane (phosphatidylserine translocation) (*19*). 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 and expressed in RFUs per well.

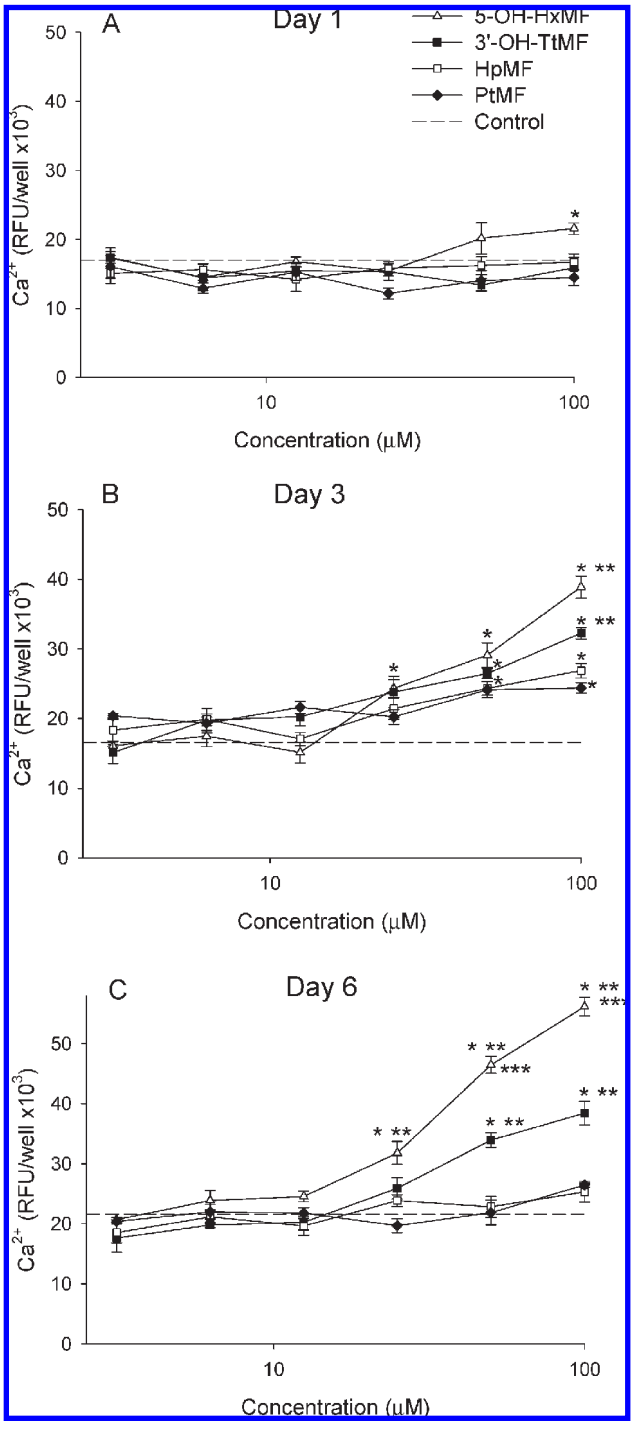

Figure 2. Effect of PMFs on intracellular Ca^{2+} levels in mature adipocytes. $[Ca^{2+}]_i$ was measured with fluo-3/AM, as described under Materials and Methods. Here and in **Figure 3**, adipocytes were treated with PMFs (3.125−100 μM) or vehicle for 1 (**A**), 3 (**B**), or 6 (**C**) days in 96-well microplates. Results are presented as relative fluorescence units (RFUs) per well (mean ± SE of quadruplicate determinations from two independent experiments). The *X*-axis is log (common). ∗ indicates statistically significant differences ($p < 0.05$) between the tested PMF and corresponding control; ∗∗, $p < 0.05$, as compared between day 1 and day 3 or 6 for the same compound and concentration; ∗∗∗, $p < 0.05$, as compared between days 3 and 6 for the same compound and concentration.

Fluorescence microscopy of Annexin V-labeled cells was employed to visualize cellular apoptotic changes. For fluorescent digital imaging, cells grown on coverslips were labeled with 1 μM Annexin V-Alexa Fluor 488 (Molecular Probes) in Annexin V binding buffer for 30 min at 37 °C.

Table 1. Effective Concentrations of PMFs in the Mature 3T3-L1 Adipocytes[a]

	increase in $[Ca^{2+}]_i$, (EC_{50}, μM)			induction of apoptosis (EC_{50}, μM)		
compd	day 1	day 3	day 6	day 1	day 3	day 6
5-OH-HxMF	48.2 ± 6.6	26.2 ± 5.2	29.2 ± 4.6	26.1 ± 1.7**	25.2 ± 5.6	14.2 ± 6.3*, **
3′-OH-TtMF	>100	30.6 ± 4.2	33.3 ± 4.0	>100	31.1 ± 2.5	13.0 ± 1.5*, **
HpMF	>100	36.1 ± 14.7	>100	>100	40.8 ± 3.5	59.2 ± 5.4*
PtMF	>100	45.9 ± 6.9	>100	>100	46.8 ± 4.3	56.1 ± 3.6

[a] EC_{50} values were calculated as described under Materials and Methods. Mean ± SE. *, $p < 0.05$, as compared between days 3 and 6 for the same compound; **, $p < 0.05$, as compared between EC_{50} for $[Ca^{2+}]_i$ increase and EC_{50} for induction of apoptosis for the same compound and time point.

The fluorescence images were acquired 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 a SuperFluor 40X 1.3 NA oil-immersion objective (Nikon) and CoolSnapFX digital CCD camera (Photometrics). Image analysis was performed using MetaFluor 7.0 software (Molecular Devices/Universal Imaging) (*13*, *29*).

Calpain and Caspase-12. Calpain activation in adipocytes was measured with the membrane-permeable fluorogenic peptide substrate t-Boc-Leu-Met-CMAC (50 μM; CMAC, 7-amino-4-chloromethyl coumarin; Molecular Probes) (*19*, *22*, *23*). The activity of the Ca^{2+}-dependent caspase-12 was measured with the fluorogenic peptide substrate Ala-Thr-Ala-Asp-AFC (50 μM; AFC, 7-amino-4-trifluoromethyl coumarin; Caspase-12 Fluorometric Assay Kit, BioVision) (*23*). The fluorescence of cells grown in 96-well microplates and loaded with the fluorogenic substrates was measured in the FLx800 reader.

Data Analysis. Statistical analyses were performed with one- or two-way ANOVA followed by multiple-comparison tests as posthoc analysis using Sigma Plot 11.0 (Systat Software). EC_{50} values of PMFs for increasing $[Ca^{2+}]_i$ and induction of apoptosis were calculated using a four-parameter logistic dose−response model (4PL function, Sigma Plot 11.0). A probability of < 0.05 ($p < 0.05$) was deemed to be statistically significant.

RESULTS

Intracellular Ca^{2+}. The basal levels of intracellular Ca^{2+} were measured in mature 3T3-L1 adipocytes treated with PMFs for 1, 3, or 6 days. PMFs induced a sustained increase of $[Ca^{2+}]_i$ in a concentration- and time-dependent fashion (**Figure 2**). Effective concentrations (EC_{50}) of PMFs are presented in **Table 1**. Hydroxylated PMFs were more effective in increasing $[Ca^{2+}]_i$, particularly at day 6 of treatment, and 5-OH-HxMF was the only tested PMF effective at day 1 of treatment. These results demonstrate that PMFs increase basal levels of intracellular Ca^{2+} in mature adipocytes in vitro. A delayed (days 1 and 3) onset of the $[Ca^{2+}]_i$ increase and its sustainability (day 6) may indicate involvement of genomic rather than nongenomic mechanisms in producing this effect (*20*).

Apoptosis. Apoptosis in the 3T3-L1 adipocytes was evaluated by the plasma membrane changes (loss of membrane asymmetry due to phosphatidylserine translocation). Mature adipocytes were treated with PMFs as described above for $[Ca^{2+}]_i$ measurements. PMFs induced apoptosis in these cells in a concentration- and time-dependent fashion similar to that observed for their $[Ca^{2+}]_i$-elevating effect (**Figure 3**). EC_{50} values of PMFs for induction of apoptosis were generally in the same range as those for inducing $[Ca^{2+}]_i$ increase (see **Table 1**). However, at day 6 of treatment, effective concentrations of hydroxylated PMFs for inducing apoptosis were significantly lower than those for increasing $[Ca^{2+}]_i$. 5-OH-HxMF was the only tested PMF effective in inducing apoptosis at day 1 of treatment, which correlates with its $[Ca^{2+}]_i$-elevating activity at this time point. A delayed onset of the PMF-induced apoptosis in adipocytes was associated with the delayed increase of $[Ca^{2+}]_i$ (compare **Figures 2B**,**C** and **3B**,**C**). Cellular apoptotic changes in adipocytes treated with PMFs are

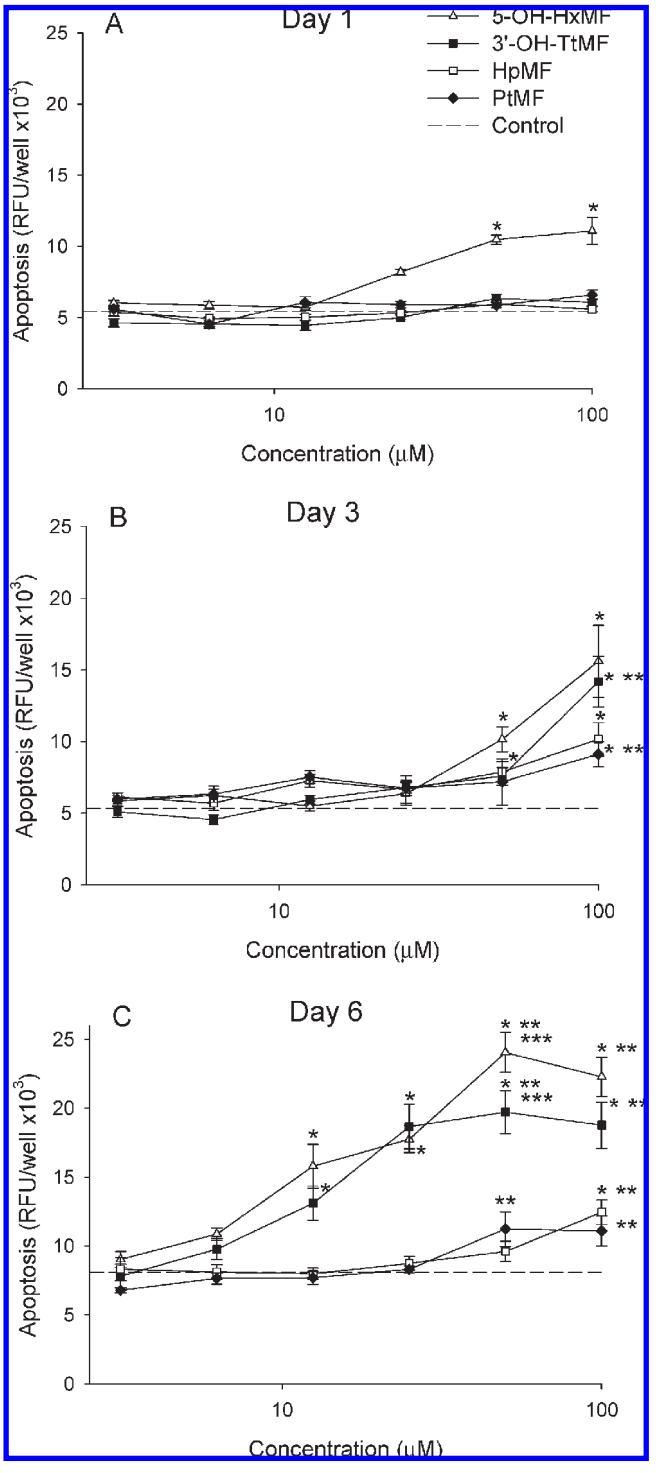

Figure 3. Apoptosis-inducing activity of PMFs in mature adipocytes. Apoptosis was measured with Annexin V-Alexa Fluor 488, as described under Materials and Methods.

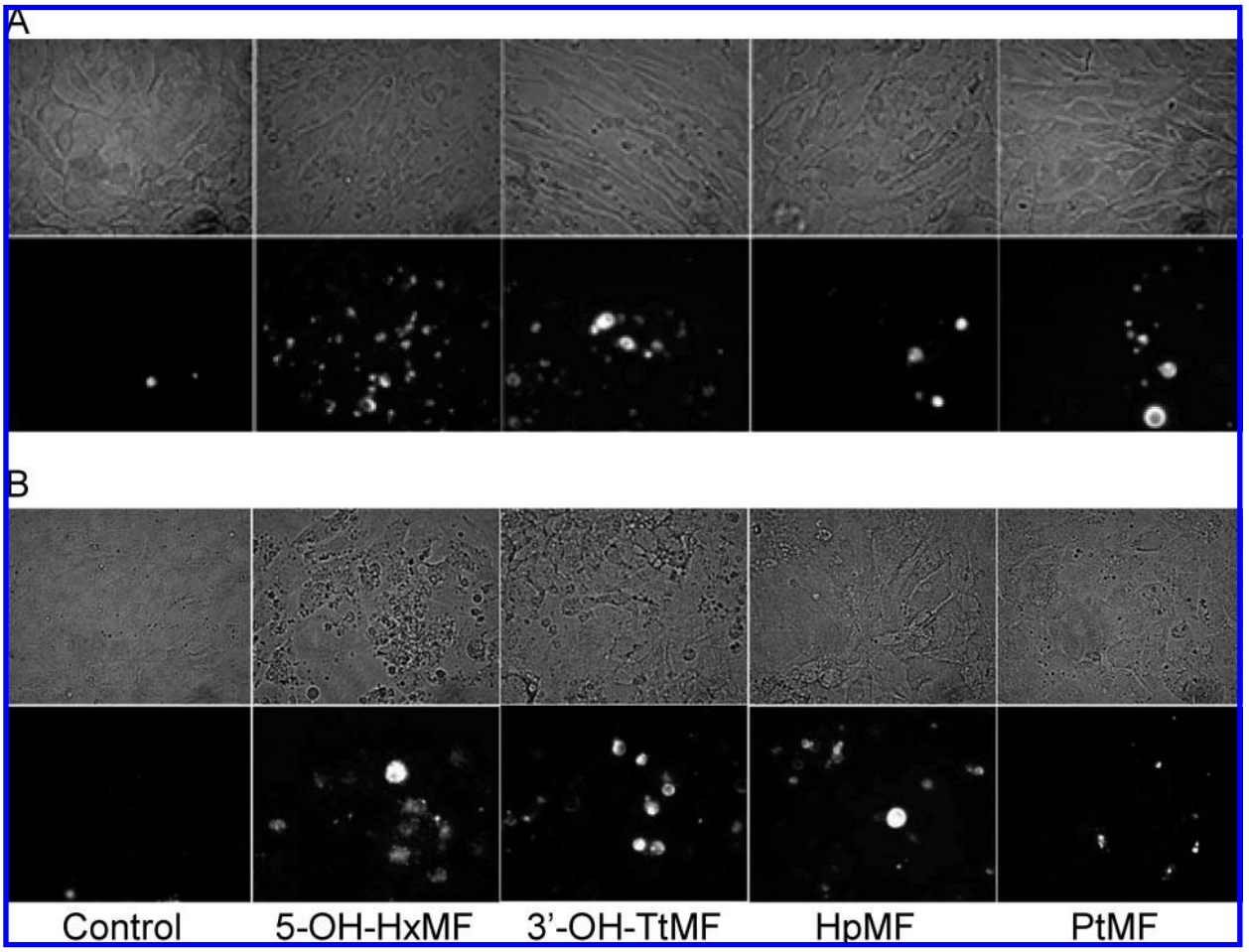

Figure 4. Cellular apoptotic changes in mature adipocytes treated with PMFs: upper rows, phase-contrast images; lower rows, fluorescence images. Adipocytes were treated with PMFs at a concentration of 50 μM for 3 (**A**) or 6 (**B**) days and labeled with Annexin V-Alexa Fluor 488, as described under Materials and Methods. Note labeling of apoptotic (round) cells.

shown in **Figure 4**. 5-OH-HxMF was the most effective PMF in inducing phosphatidylserine translocation in the plasma membrane (Annexin V assay) and DNA condensation/nuclear fragmentation.

Calpain and Caspase-12. A sustained increase in $[Ca^{2+}]_i$ in mature 3T3-L1 adipocytes treated with PMFs (100 μM) for 1, 3, or 6 days was accompanied by activation of the Ca^{2+}-dependent apoptotic proteases, μ-calpain and caspase-12, as evaluated with the specific fluorogenic peptide substrates (**Figure 5**). Hydroxylated PMFs (5-OH-HxMF and 3′-OH-TtMF) were significantly more active in inducing calpain and caspase-12 activity than nonhydroxylated PMFs (HpMF and PtMF). 5-OH-HxMF (100 μM) was the only tested PMF increasing calpain and caspase-12 activities at day 1 of treatment, which correlates with its effects on $[Ca^{2+}]_i$ and apoptosis at this concentration and time point. The PMF-induced calpain activity reached maximum at day 3 of treatment followed by a statistically significant decrease at day 6, whereas caspase-12 activity reached maximum at day 6. These findings support our previous observations (*20*, *23*) that calpain activation precedes caspase-12 activation in the Ca^{2+}-mediated apoptotic signaling pathway and, thus, may be necessary for caspase-12 activation.

DISCUSSION

Obesity is associated with an increased adipocyte number and size, leading to the excessive accumulation of adipose tissue (*8*−*10*). Apoptosis, a highly regulated form of cell death, is the main mechanism for controlling cell number in most tissues (*30*). However, studies on the role of apoptosis in fat tissue have been limited by the fact that mature adipocytes are extremely stable and not thought to be capable of undergoing apoptosis. As a consequence, our understanding of the apoptotic signaling pathways in adipose tissue is limited.

Induction of the death of adipocytes through apoptosis may emerge as a promising strategy for the prevention and treatment of obesity because removal of adipocytes via this mechanism will result in reducing body fat. Recent data suggest that flavonoids such as resveratrol (*7*) and the prenylflavonoid from hop, xanthohumol (*6*), exhibit apoptosis-inducing activity in adipocytes; however, the mechanisms involved remain obscure. In vitro and in vivo antiobesity effects of phenolic compounds and their molecular signaling mechanisms were recently reviewed (*5*). It was noted that antiobesity properties of epigallocatechin-3-gallate, esculetin, gallic acid, quercetin, and genistein could be associated with the induction of adipocyte apoptosis.

Previous studies with PMFs demonstrated their pro-apoptotic effects in several tumor cell lines, and these effects were associated with increased Ca^{2+} activation of calpain and caspase-12 (*12*). In this study, we investigated the apoptotic effects of PMFs in mature mouse 3T3-L1 adipocytes and examined the mechanism of Ca^{2+}-mediated apoptosis in these cells. The results obtained demonstrate that PMFs are effective in increasing $[Ca^{2+}]_i$ and inducing Ca^{2+}-mediated apoptosis in mature adipocytes. These observations are important because they indicate that apoptosis

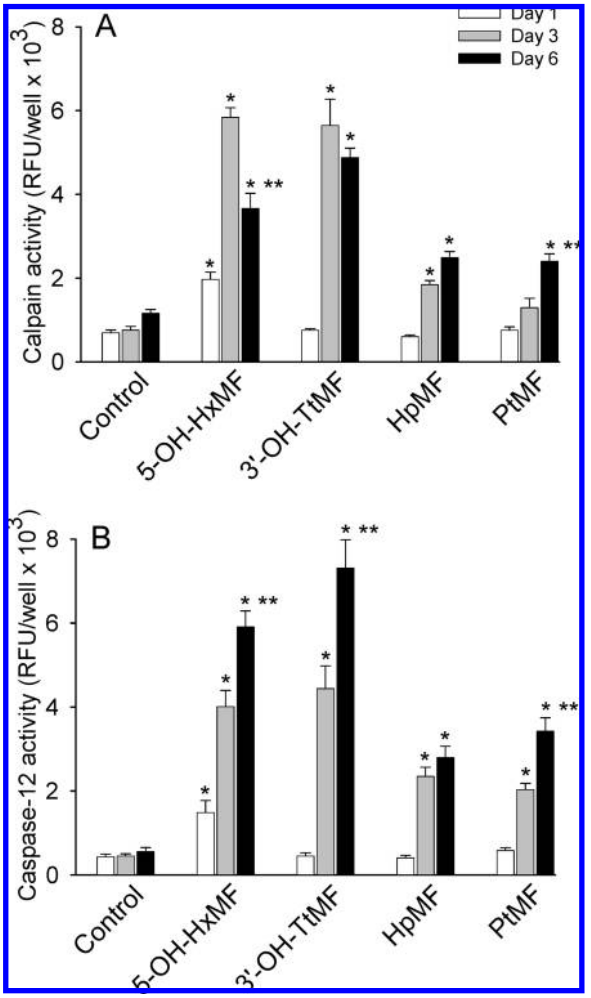

Figure 5. Calpain and caspase-12 activities in mature adipocytes treated with PMFs. Adipocytes were treated with PMFs (100 μM) or vehicle for 1, 3, or 6 days. Calpain and caspase-12 activities were measured with the specific fluorogenic peptide substrates, as described under Materials and Methods. $*$ indicates statistically significant differences ($p < 0.05$) between the tested PMF and corresponding control; $**$, $p < 0.05$ between day 1 and day 3 or 6 of the treatment; $***$, $p < 0.05$ between days 3 and 6 of the treatment.

can be induced in mature, differentiated adipocytes and that induction of apoptosis in these cells is associated with a sustained increase in intracellular Ca^{2+}. Although involvement of PMFs in increasing $[Ca^{2+}]_i$ and inducing apoptosis has been previously demonstrated in several cancer cell lines (*12*−*15*), the findings presented here demonstrate, for the first time, that the PMF-induced Ca^{2+}-mediated signaling can be employed to trigger apoptosis in adipocytes. Furthermore, our data provide the identification of apoptotic molecular targets in these cells, calpain and caspase-12. Taken together, the findings obtained with adipocytes and cancer cells demonstrate that Ca^{2+}-mediated apoptosis appears to be a universal mechanism of apoptotic cell death in such different cell types because cellular Ca^{2+} can act as an apoptotic initiator and directly recruit Ca^{2+}-dependent apoptotic effectors capable of executing apoptosis.

Hydroxylated PMFs were significantly more effective in increasing $[Ca^{2+}]_i$ and inducing apoptosis in adipocytes, which may be attributed to their stimulating effect on the production of reactive oxygen species (ROS) (*14*). ROS have been shown to induce Ca^{2+} influx and accelerate the plasma membrane Ca^{2+} channel opening, and the endoplasmic reticulum Ca^{2+} release channels are redox-regulated (*31*). However, it is probably more important to consider that elevation of intracellular Ca^{2+} levels is responsible for the activation of ROS-generating enzymes and the formation of free radicals by the mitochondrial respiratory chain (*32*, *33*), which, in turn, contribute to activation of the apoptotic signaling pathways. It is noteworthy that hydroxylated PMFs were more effective in inducing apoptosis in human breast cancer cells (*12*, *13*) than in adipocytes, and apoptosis-inducing activity of hydroxylated PMFs in cancer cells was observed at markedly lower concentrations. These findings suggest that the hydroxyl group enhances the apoptosis-inducing activity of PMFs and that cell-type specificity of this effect may derive from the differences in the cellular Ca^{2+} regulatory mechanisms.

Collectively, results reported here support the hypothesis that PMFs evoke apoptosis in mature adipocytes via activation of the Ca^{2+}/calpain/caspase-dependent pathway. Further studies are warranted to explore the potential for targeting Ca^{2+}-mediated apoptosis as a therapeutic approach for the induction of adipocyte death. In this context, dietary PMFs may serve as an effective strategy for the prevention and treatment of obesity.

ABBREVIATIONS USED

PMFs, polymethoxyflavones; 5-OH-HxMF, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone; 3′-OH-TtMF, 3′-hydroxy-5,6,7,4′-tetramethoxyflavone; HpMF, 3,5,6,7,8,3′,4′-heptamethoxyflavone; PtMF, 5,6,7,3′,4′-pentamethoxyflavone; $[Ca^{2+}]_i$, intracellular calcium level; IBMX, 3-isobutyl-1-methylxanthine; DMSO, dimethyl sulfoxide; RFUs, relative fluorescence units.

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