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Involvement of Ras/Raf-1/ERK Actions in the Magnolol-Induced Upregulation of p21 and Cell-Cycle Arrest in Colon Cancer Cells

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Previously, we showed that magnolol induces cell-cycle arrest in cultured colon and liver cancer cells through an upregulation of the p21 protein [1]. The aim of this study was to delineate the molecular mechanism underlying this magnolol-induced increase of p21 protein. Thus our RT-PCR analysis demonstrated that the mRNA levels of p21 were increased at 1 h after magnolol treatment and sustained for at least 24 h. The p21 promoter activity was also increased by magnolol treatment. Western blot analysis demonstrated that treatment of COLO-205 cells with magnolol increased the levels of phosphorylation of extracellular signal-regulated kinase (ERK). Pretreatment of the cells with PD98059 abolished the magnolol-induced upregulation of p21 protein, suggesting the involvement of an ERK pathway in the magnolol-induced upregulation of p21 in COLO-205 cells. Ras inhibitor peptide abolished the magnolol-induced increase of phosphorylated ERK protein levels, increase of p21 protein, and decrease of thymidine incorporation. Moreover, treatment of COLO-205 with magnolol increased the phosphorylated Raf-1 protein (the Ras target molecule). Pretreatment of the cells with Raf-1 inhibitor reversed the magnolol-induced decrease in thymidine incorporation. Treatment of the cells with CaM kinase inhibitor, but not protein kinase A (PKA) inhibitor or phosphatidylinositol 3-kinase (PI3K) inhibitor, abolished the magnolol-induced activation of ERK and decrease of thymidine incorporation. Taken together, our results suggest that magnolol activates ERK phosphorylation through a Ras/Raf-1-mediated pathway. Subsequently, p21 expression is increased, and finally thymidine incorporation is decreased.

Key words: Ras; Raf; ERK; p21; colon cancer

INTRODUCTION

To identify medicinal agents capable of retarding the cell cycle and/or activating the cellular apoptotic response in cancerous cells has been the focus most recently of experimental and clinical investigators in searching for new therapeutic strategies. Previously, we have demonstrated that magnolol, a hydroxylated biphenyl compound isolated from Chinese herb Hou po's of *Magnolia officinalis*, can suppress proliferation of cultured human colon and liver cancer cells by inhibiting DNA synthesis and activating apoptosis in vitro and in vivo [1]. Magnolol at low doses (3–10 μM) inhibited DNA synthesis and decreased cell number in cultured human liver (Hep-G2 and Hep-3B) and colon (COLO-205 and HT-29) cancer cell lines in a dose-dependent manner, but not in human untransformed cells such as keratinocytes, fibroblasts, and human umbilical vein endothelial cells (HUVEC). When magnolol concentration was increased to 100 μM, apoptosis was observed in COLO-205 and Hep-G2 cells, but not in cultured human fibroblasts and HUVEC. The magnolol-induced cell-cycle arrest occurred when the cyclin-dependent kinase 2 (CDK2) was inhibited, just as the protein level of p21 was increased. The magnolol-induced apoptosis, however, was associated with a sequence of intracellular events including (a) increased cytosolic free Ca^{2+};

Abbreviations: CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; CDK, cyclin-dependent kinase; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenases; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C.

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(b) increased translocation of cytochrome c from mitochondria to cytosol; (c) downregulation of bcl-2 protein; and (d) activation of caspases 3, 8, and 9 [2].

Although the elevation of p21 protein levels has been suggested to be responsible for magnolol-induced cell-cycle arrest, the molecular mechanism underlying the magnolol-induced increase of p21 expression is still unclear. The *p21* gene is a target for diverse signals causing cell growth arrest and differentiation. Introduction of p21 protein to human untransformed and cancer cells induces cell-cycle arrest [3–5]. P21 arrests the cell cycle through binding and inactivating the CDK system [4,5]. A p21 mutation, which specifically abrogates its binding to CDKs, was identified in a primary breast tumor [6], suggesting that p21 exerts tumor suppressor properties.

It has been proposed that a mitogen-activated protein kinase (MAPK)-mediated pathway might be a key regulator of cell proliferation and differentiation in some multicellular organisms [7,8]. Over-activity of the MAPK-mediated pathway is oncogenic in a variety of cell types [9,10]. Conversely, functional deficiency of the MAPK-mediated pathway has been associated with certain developmental abnormality [11]. However, a recent report also showed that activation of the MAPK pathway by Raf-1 family protein kinases could cause cell-cycle arrest by induction of p21 protein in mouse fibroblasts [12]. Furthermore, the MAPK pathway has been implicated in the p21 upregulation and cell-cycle arrest mediated by the transforming growth factor-β in human keratinocyte cells [13].

In the present study, we found that the Ras/Raf-1/ERK pathway is involved in the magnolol-induced upregulation of p21 protein in the COLO-205 cells. The details of these experiments are described below and shed new light on the molecular mechanisms involved in magnolol-induced inhibition of colon cancer growth.

MATERIALS AND METHODS

Reagents

H89 and Kn62 were purchased from Biomal (Plymouth Meeting, PA). Magnolol was purchased from Pharmaceutical Industry, Technology and Development Center (Taipei Country, Taiwan). Antisera against p21, extracellular signal-regulated kinase (ERK), Ras, Raf, PKCη and PKCθ monoclonal antibodies were obtained from Transduction (San Diego, CA). Antisera against PKCα, β, γ, ε, δ, and ζ polyclonal antibodies were purchased from Gibco BRL Life Technologies Inc. (Grand Island, NY). Anti-glyceraldehyde-3-phosphate dehydrogenases (G3PDH) monoclonal antibodies were purchased from Biogenesis (Kingston, NH). Nitro-blue-tetrazolium chloride/bromo-chloro-3-indolyl-phosphate was obtained from Kirkegaard Perry Laboratory (Gaithersburg, MD). Ras inhibitor peptide, (VPPPVPPRRR), and PD98959 were purchased from Sigma (St. Louis, MO). Staurosporine, RO32-0432, Raf inhibitor, and Wortmannin were purchased from Merck (Parmstadt, Germany). The concentration of PD 98059, staurosporine, RO32-0432, Raf inhibitor, Ras inhibitor, H89, Kn62, and Wormannin used in this study was 100 μM, 100 nM, 10 nM, 5 μM, 100 nM, 10 nM and 5 μM, respectively (around 5–10 times of their IC_{50}). The concentrations of these inhibitors used in this study did not cause any cytotoxicity as evidenced by no significant change in thymidine incorporation when the cells were treated with inhibitors alone.

Cell Culture

COLO-205 cell line (CCL-222; American Type Culture Collection) originating from a poorly differentiated human colon adenocarcinoma was used in this study. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 μg/mL), and glutamine (0.3 mg/mL) in a humidified incubator (37°C, 5% CO_2). Magnolol in 0.1% dimethyl sulfoxide (DMSO) was added at the indicated doses. For control specimens, the equivalent volume of the 0.1% DMSO without magnolol was added.

[^{3}H]Thymidine Incorporation

As previously described [1], COLO-205 at a density of 1×10^4 cells/cm^3 were applied to 24-well plates in growth medium (RPMI 1640 plus 10% FBS). After the cells had grown to 70%–80% confluence, they were rendered quiescent by incubation for 24 h in RPMI 1640 containing 0.04% FBS. Then RPMI 1640 supplemented with 10% FBS and 0.05% DMSO (control) or various concentrations of magnolol in 0.05% DMSO was added to the cells and the cultures were allowed to incubate for 18 h. During the last 3 h of the incubation with or without magnolol, [^{3}H]thymidine was added at 1 μCi/mL (1 μCi = 37 kBq). Incorporated [^{3}H]thymidine was extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

Protein Extraction and Western Blot Analysis

The cells were seeded onto 100-mm dishes and grown in RPMI 1640 supplemented with 10% FCS. After the cells had grown to subconfluence, magnolol in 0.05% DMSO or 0.05% DMSO with magnolol was added to the cells at various concentrations. The cells were washed with phosphate-buffered saline (PBS) and then lysed in lysis buffer (Tris HCl 0.5 M, pH 6.8; SDS 0.4%). Western blot analysis was performed as previously described [2,14]. The protein samples (50 μg per lane) were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel electrophoresis was performed at constant 120 V for 3–4 h at 16–18°C. After protein separation, each gel was transferred

onto an Immobilion-P membrane. Immunodetection was carried out by probing with proper dilutions of specific antibodies at 4°C for overnight. The primary antibodies were used at a concentration of 1:1,000 dilution. The secondary antibodies, alkaline phosphatase-coupled anti-mouse or anti-rabbit antibodies purchased from Jackson (Westgrove, PA), were incubated at room temperature for 1 h at a concentration of 1:5,000 or 1:1,000 dilutions, respectively. The specific protein complexes were identified with nitro-blue-tetrazolium chloride/bromo-chloro-3-indolyl-phosphate. In each experiment, membranes were also probed with anti-G3PDH antibody to correct for difference in protein loading. To study whether protein kinase C (PKC) or MAPK pathways were involved in the magnolol-induced upregulation of p21, the COLO-205 cells were pretreated with staurosporine (100 nM) or PD98059 (50 and 100 μM) for 30 min and followed by treatment with 50 μM magnolol.

Subcellular Fractionation

The COLO-205 cells were collected by centrifugation at 200 g for 5 min at 4°C and washed once with ice-cold PBS. The cells were partitionated into the cytosolic and the particulate portion according to the previously described method [15]. Briefly, the cells were suspended in extraction buffer, containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and protease inhibitors. After 30 min incubation on ice, the cells were homogenized with a glass Dounce and a B pestle. Cell homogenates were spun at 14,000*g* for 15 min and supernatants were removed as cytosolic portion. The pellets (particulate portion) were lysed for Western blot analysis. To confirm that the pellets were not contaminated with cytosolic proteins, Western blot analysis of α tubulin was conducted.

RT-PCR

Total RNAs were isolated from DMSO- or magnolol-treated COLO-205 cells and the cDNAs were prepared with reverse transcription (RT)-polymerase chain reaction (PCR) technique as previously described [1]. The p21 cDNA amplication was performed by incubating 20 ng equivalents of cDNA in 100 mM Tris-HCl buffer, pH 8.3, containing 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 200 μM concentration of each dNTP, and 50 U/mL of Super Taq DNA polymerase with the following oligonucleotide primers: 5′-AGGAGGCCCGTGAGCGAGCGATGGAAC-3′ and 5′-ACAAGTGGGGAGGAAGTAGC-3′. The cDNA sequence of β-actin was also amplified as a control in the same method with the following primers: 5′-GATATCGCCGCGCTCGTCGTCGAC-3′ and 5′-CAGGAAGGAAGGCTGGAAGAGTGC-3′. Thermal cycle conditions were as follows: 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, and 1 cycle at 72°C for 10 min (for p21); 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 45 s, 65°C for 45 s, 72°C for 2 min, and 1 cycle at 72°C for 10 min (for GAPDH). PCR products were analyzed on 1.8% agarose gels.

Plasmid Transfection and Reporter Assays

Plasmid pWWP-luc containing the promoter of human p21 between positions −2,300 and +8 was a gift from Vogelstein (Johns Hopkins University, Baltimore, MD) [3]. The p21 promoter region was removed with HindIII and subcloned into the unique HindIII site present within the pGL3 plasmid (Promega, Madison, WI). The identity of the sequences was confirmed with an ABI PRISM 377 DNA Analysis System (Perkin-Elmer, North Point, Hong Kong).

For the p21 reporter activity assay, COLO-205 cells were seeded in 6-well plates at a density of 5×10^5 cells/well. In brief, cells were transiently transfected with 1.1 μg of plasmid DNA containing 0.1 μg of the Renilla luciferase construct, phRL-TK (Promega), to control transfection efficiency and 1 μg of the appropriate p21 promoter firefly luciferase (FL) construct. The next day, cells were transfected with pGL3/hp21 and phRL-TK (internal control plasmid) using the Transfast™ (Promega). After transfection (1 h), the medium was replaced with complete medium, and incubation continued for another 42 h. Transfected cells were then treated with magnolol at a concentration of 50 μM for 6 h, and cell lysates were collected. Luciferase activities were recorded in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) with the dual luciferase assay kit (Promega) according to the manufacturer's instructions. Luciferase activities of reported plasmids were normalized to luciferase activities of the internal control plasmid.

RESULTS

Magnolol Upregulates p21 mRNA Expression and Increases p21 Promoter Activity in COLO-205 Cells

Previously, we have demonstrated that magnolol induces cell growth inhibition in colon cancer cells through an upregulation of the p21 protein. To determine whether transcriptional regulation was involved in the magnolol-induced increase in p21 protein, RT-PCR analysis was conducted. As illustrated in Figure 1a, the magnolol (50 μM)-induced upregulation of p21 mRNA levels was observed at 1 h after treatment and lasted for more than 24 h, suggesting that magnolol treatment caused elevation of p21 protein levels by increased transcription. Figure 1b shows that magnolol (50 μM) treatment resulted in the upregulation of the p21 promoter

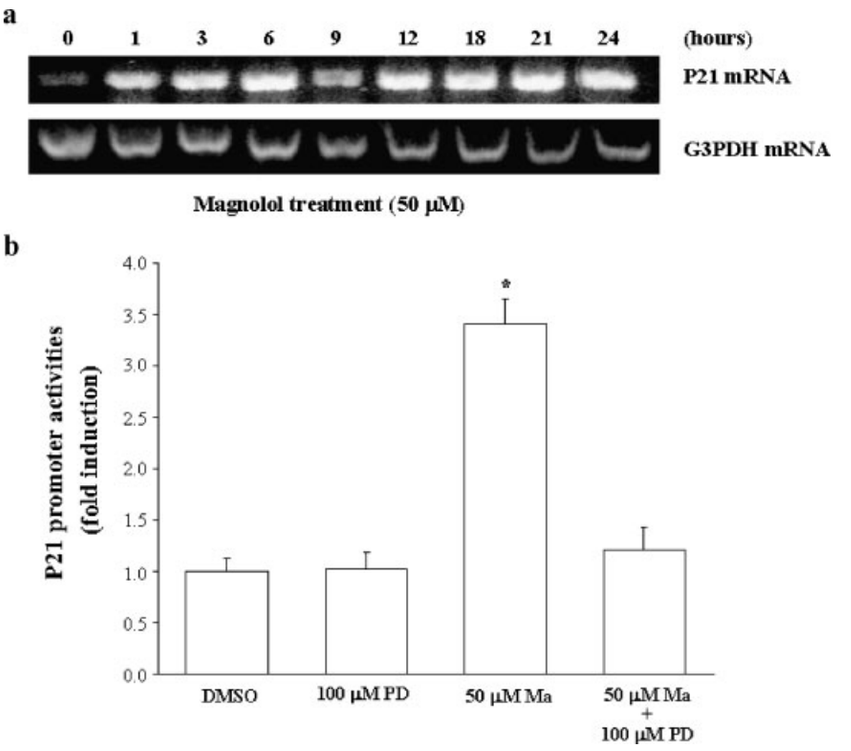

Figure 1. Magnolol increases p21 mRNA expression and p21 promoter activity in COLO-205. (a) The increased p21 mRNA levels in COLO-205 were observed after 1 h treatment with magnolol (50 μM) and lasted for at least 24 h. (b) Treatment of COLO-205 with magnolol (50 μM) for 6 h resulted in an increase in p21 promoter activity, which could be prevented by pretreatment of the cells with an ERK inhibitor, PD98059 (100 μM). Values represent the mean ± SEM ($n = 4$). Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *Ma-treated group different from control. Ma, magnolol; PD, PD98059.

activity by 3.5-fold. Pretreatment of the cells with PD98059 (an ERK inhibitor) at a dose of 100 μM abolished the magnolol-induced upregulation of p21 promoter activity.

Involvement of ERK Activation in the Magnolol-Mediated Increase of p21 Upregulation in COLO-205 Cells

Because activation of ERK has been implicated in the regulation of p21, we studied the molecular mechanism underlying magnolol-induced upregulation of p21 by examining the levels of phosphorylated ERK (pERK) protein. As illustrated in Figure 2a, an increase of pERK protein was observed in COLO-205 after 40-min treatment with magnolol (50 μM). Pretreatment of the cells with 100 μM PD98059 abolished the magnolol-induced upregulation of p21 protein (Figure 2b) and inhibition of thymidine incorporation (Figure 2c). As shown in our previous studies [1], the concentration (50 μM) of magnolol used in this study did not cause any apoptotic response in COLO-205.

Involvement of Ras in the Magnolol-Induced ERK Activation in COLO-205 Cells

We further investigated the involvement of Ras molecule in the magnolol-induced activation of ERK in COLO-205 cells. Treatment of COLO-205 cells with Ras inhibitor peptide abolished the magnolol-induced increases of the protein levels of phosphorylated ERK (Figure 3a) and p21 (Figure 3b), and prevented the magnolol-induced decrease of thymidine incorporation (Figure 3c). The activation of Raf molecule, which is a downstream molecule of Ras, was also observed in the magnolol-treated COLO-205 cells (Figure 4a). Moreover, pretreatment of COLO-205 cells with Raf inhibitor (10 nM) prevented the magnolol-induced decrease of thymidine (Figure 4b).

Involvement of CaM Kinase Activation on the Magnolol-Induced Increase of p-ERK

Previously, we have demonstrated that treatment of the COLO-205 with staurosporine for 18 h abolished the magnolol-mediated increase of p21 protein level [1], suggesting that PKC might be involved in the magnolol-induced cell-cycle arrest. Because staurosporine is a broad-specificity kinase inhibitor, which may alter other kinases besides PKC, we further examined the involvement of PKC in the regulation of magnolol-induced cell-cycle arrest in COLO-205. Initially, we examined the translocation

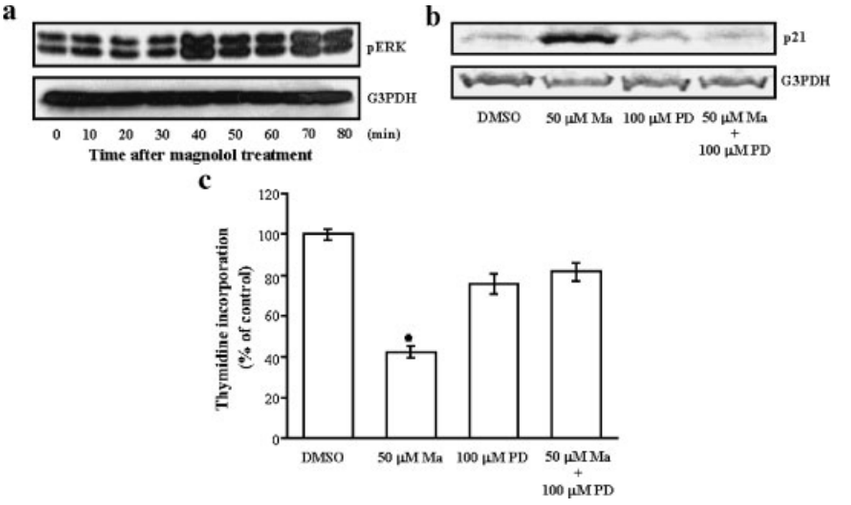

Figure 2. Involvement of ERK activation in the magnolol-induced increases of p21 protein and inhibition of thymindine incorporation in COLO-205. (a) The magnolol-induced increases of the phosphorylated ERK protein were observed at as early as 40 min after magnolol (50 μM) treatment. Membrane was probed with anti-G3PDH antibody to verify equivalent loading. Pretreatment of the cells with PD98059 (100 μM), abolished the magnolol-induced increases of p21 protein (b), and decreases of thymidine incorporation (c). The protein extraction for p21 and G3PDH detection was done at 24 h after drug treatment. Results from a representative experiment are shown. Values represent the mean ± SEM ($n = 3–4$). Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *Ma-treated group different from control group and Ma + PD-treated group. Ma, magnolol; PD, PD98059.

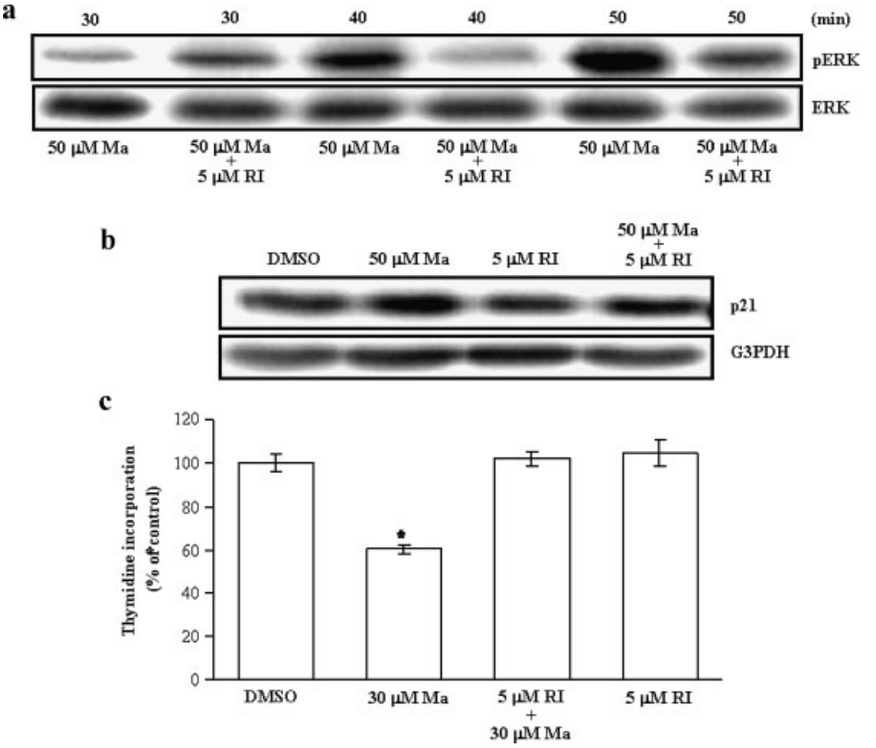

Figure 3. Involvement of Ras activation in the magnolol-induced ERK phosphorylation and inhibition of thymindine incorporation in COLO-205. Pretreatment of COLO-205 with Ras inhibitor abolished magnolol-induced increases of the phosphorylated ERK protein (a) and p21 protein (b), and decreases of thymidine incorporation (c). The proteins were extracted from COLO-205 treated with magnolol or vehicle for 24 h. Membrane was probed with anti-total ERK or G3PDH antibody to verify equivalent loading. Results from a representative experiment are shown. Values represent the mean ± SEM ($n = 3–4$). Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *Ma-treated group different from control group and Ma + RI-treated group. Ma, magnolol; RI, Ras inhibitor.

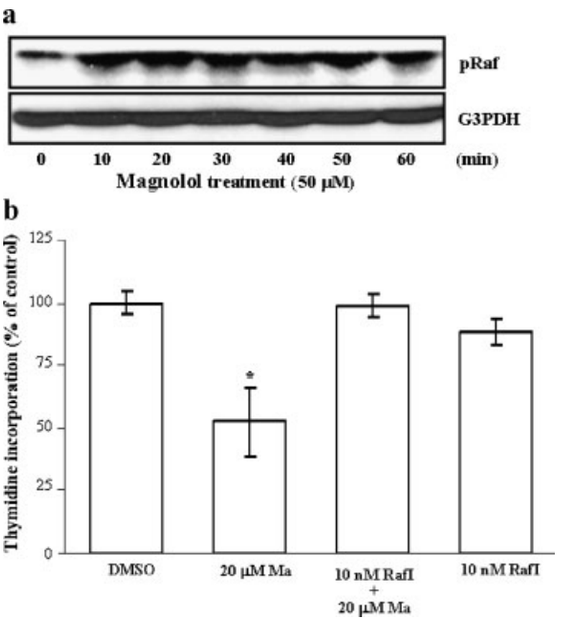

Figure 4. Involvement of Raf activation in the magnolol-induced inhibition of thymidine incorporation in COLO-205 cells. (a) The levels of phosphorylated Raf protein were increased in the COLO-205 after 10-min treatment with magnolol (50 μM). Membrane was probed with anti-G3PDH antibody to verify equivalent loading. (b) Pretreatment of the COLO-205 cells with a Raf-specific inhibitor prevented the magnolol-induced inhibition of thymidine incorporation. Values represent the mean ± SEM ($n = 3–4$). Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *Ma-treated group different from control and Ma + RafI-treated group. Ma, magnolol; RafI, Raf inhibitor.

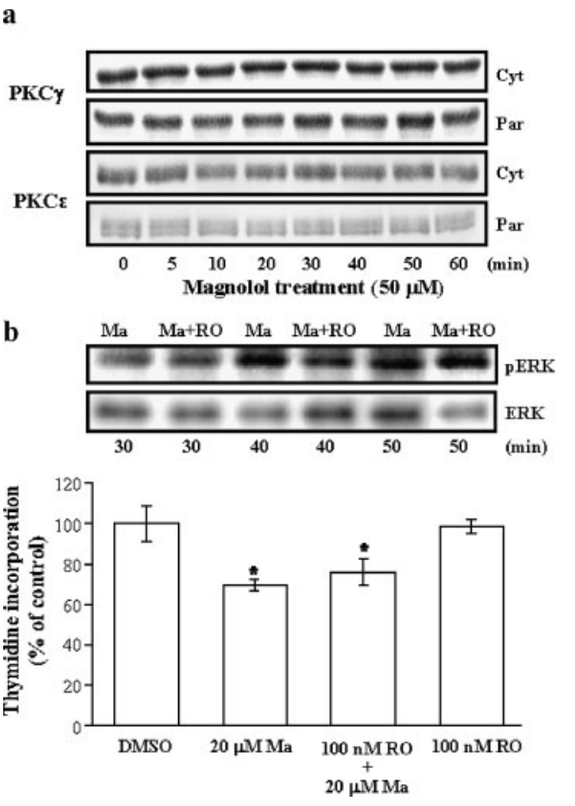

Figure 5. PKC is not involved in the magnolol-induced cell-cycle arrest in COLO-205 cells. (a) Treatment of the COLO-205 cells with magnolol (50 μM) for 1 h did not cause a significant translocation of PKCγ or PKCε from the cytosolic fraction to membrane fraction. (b) Pretreatment of the COLO-205 cells with a PKC-specific inhibitor, RO32-0432, could not prevent the magnolol-induced upregulation of pERK and inhibition of thymidine incorporation. Values represent the mean ± SEM. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *Ma- and Ma + RO-treated group different from control group. Ma, magnolol; RO, RO32-0432.

of PKC from cytosol to membrane and showed that treatment of COLO-205 with magnolol for 1 h did not cause any significant translocation of PKCγ or PKCε (Figure 5a), the only two isoforms detected in the COLO-205 cells. Moreover, pretreatment of the cells with PKC-specific inhibitor (RO32-0432) could not prevent the magnolol-induced increase in the levels of pERK protein and decrease in thymidine incorporation (Figure 5b), suggesting that PKC activation might not be involved in the magnolol-induced inhibition of thymidine incorporation in COLO-205. We examined further the involvement of several other staurosporine-sensitive kinases in magnolol-induced cell-cycle arrest. As shown in Figure 6a and b, neither H89 (a protein kinase A (PKA) inhibitor) nor Wortmannin (a phosphatidylinositol 3-kinase (PI3K) inhibitor) could prevent the magnolol-induced increase in the levels of pERK protein and inhibition of thymidine incorporation. On the other hand, pretreatment of COLO-205 with Kn62 (a Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) inhibitor) prevented the magnolol-induced increase of pERK protein levels (Figure 7a) and decrease of thymidine incorporation (Figure 7b).

DISCUSSION

Previously, we have shown that magnolol induces cell-cycle arrest at the G_0/G_1 phase in colon cancer cells through an upregulation of the cyclin-dependent kinase inhibitor p21 protein. The present study was undertaken to further delineate the molecular mechanism of the magnolol-induced p21 upregulation in COLO-205 cells. It has been demonstrated that high-intensity ERK signaling leads to p21-mediated growth arrest in rodent fibroblasts [12,16] and human tumor cells [17]. In the present study, our in vitro data show that administration of magnolol at a concentration of 50 μM induced an increase of the phosphorylated ERK protein level in COLO-205 cells, indicating an activation of ERK protein. Pretreatment of the cells with PD98059 prevented magnolol-induced increase of p21 protein level, activation of p21 promoter activity and decrease of thymidine

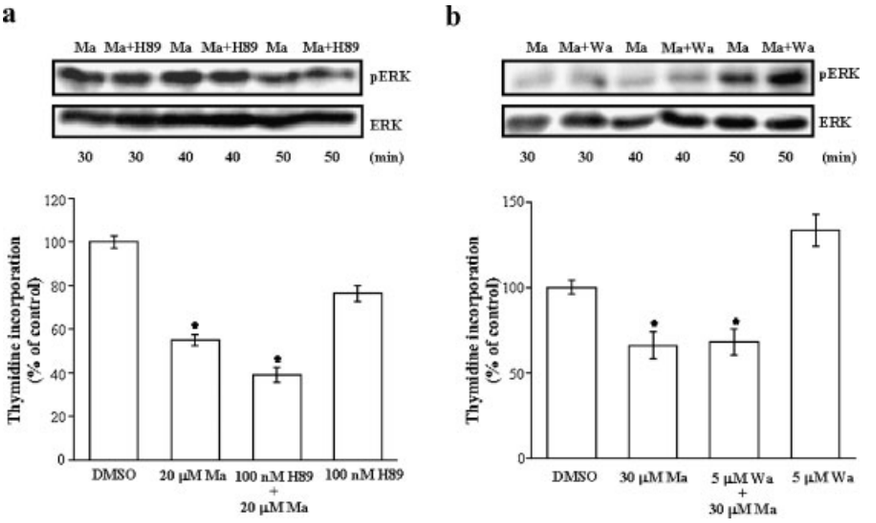

Figure 6. PKA and PI3K are not involved in the magnolol-induced cell-cycle arrest in COLO-205 cells. Pretreatment of the COLO-205 cells with a PKA inhibitor, H89 (a), or a PI3K inhibitor, Wa (b), could not prevent the magnolol-induced upregulation of pERK and inhibition of thymidine incorporation. Values represent the mean ± SEM. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *Ma-, Ma + H89- or Ma + Wa-treated group different from control group. Ma, magnolol; Wa, Wortmannin.

incorporation in COLO-205 cells (Figure 2), suggesting that the ERK-mediated signaling pathway is involved in the magnolol-induced p21 upregulation, which in turn caused cell-cycle arrest.

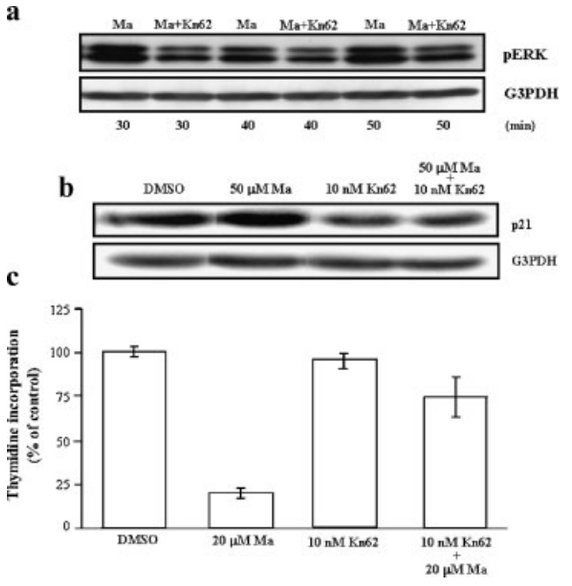

Figure 7. Involvement of CaM kinase activation on the magnolol-induced cell-cycle arrest in COLO-205 cells. Pretreatment of COLO-205 cells with CaM kinase inhibitor (Kn62) at a concentration of 10 nM prevented magnolol-induced increases of the phosphorylated ERK protein (a) and p21 protein (b), and decreases of thymidine incorporation (c). Values represent the mean ± SEM. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *Magnolol-treated group different from control group. Ma, magnolol.

Activation of the Ras/Raf signal transduction pathway has been shown to contribute to oncogenic transformation by influencing the cell-cycle machinery in numerous types of cancer [12,18]. On the other hand, it seems that Ras signaling elevates p21 to levels that arrest cell progression by means of the Raf/MAPK pathway [18,19]. Introduction of activated Ras into primary rodent and human cells induces increase of p21 protein levels, which in turn causes cell-cycle retardation at the G_0/G_1 phase [20–22]. Ras-induced elevation of p21 has been attributed to transcriptional activation [12,16,20,21] or post-translational regulation [23]. Accordingly, we examined whether a Ras-mediated pathway is involved in the magnolol-induced cell-cycle arrest in COLO-205. In the present study, we demonstrated that magnolol treatment resulted in an upregulation of p21 mRNA and an increase in p21 promoter activity (Figure 1), suggesting that transcription regulation is involved in the magnolol-induced increases of p21 protein level. Pretreatment of COLO-205 with Ras inhibitor abolished the magnolol-induced activation of ERK, together with the associated upregulation of p21 protein, and inhibition of thymidine incorporation (Figure 3). Moreover, magnolol increased activation of Raf-1, which has been indicated to be the target molecule of the activated Ras. Administration of Raf-1 inhibitor blocked the magnolol-induced inhibition of thymidine incorporation (Figure 4). Taken together, these results suggest that magnolol activated the Ras/Raf-1/ERK pathway, which in turn upregulated p21 protein through transcriptional regulation, and ultimately caused cell-cycle arrest.

Based on the finding that pretreatment of COLO-205 with staurosporine blocked the magnolol-induced upregulation of p21 protein, we previously proposed that a PCK pathway might be involved in the magnolol-induced p21 induction in COLO-205 [1]. In the present studies, however, we showed that translocation of PKCγ and PKCε (the only two PKC isoforms detected in COLO-205) from the cytosolic fraction to membrane fraction was not observed after 1 h magnolol treatment (Figure 5a), suggesting that PKC might be involved in some magnolol-mediated effects other than cell-cycle arrest. To confirm this hypothesis, we showed that pretreatment of COLO-205 with a PKC-specific inhibitor, RO32-0432, could not prevent the magnolol-induced increase the levels of pERK protein and downregulation of thymidine incorporation (Figure 5b). Because staurosporine is a broad-specificity kinase inhibitor, it is most likely that staurosporine may alter other kinases besides PKC. Indeed, we examined further the involvement of several other staurosporine-sensitive kinases in magnolol-induced cell-cycle arrest, and found that pretreatment of COLO-205 with Kn62 (a CaM kinase inhibitor), but not H89 (a PKA inhibitor) or Wortmannin (a PI3K inhibitor), prevented the magnolol-induced increase of pERK levels and decrease of thymidine incorporation (Figures 6 and 7). These data suggest that CaM kinase might be involved in the magnolol-induced cell-cycle arrest. CaM kinase is positively regulated by intracellular Ca^{2+} concentration [24], and activation of CaM kinase can activate the downstream signaling pathway including Ras family signal pathway [25].

Although our data strongly suggest that the ERK-mediated pathway is involved in the regulation of magnolol-induced increase in p21 protein in COLO-205 cells, we do not rule out the possibility that other signaling pathways might also contribute to upregulate p21 expression in the magnolol-treated COLO-205 cells. In fact, it has been indicated that the transcription of *p21* gene is regulated by multiple proteins, many of which (such as p53, *c-myc*, and Rb) are involved in the pathogenesis of cancer. Our previous studies showed that magnolol decreases the DNA synthesis in both p53 wild-type cell lines (COLO-205 and Hep-3B) and p53 mutated type cell lines (HT-29 and Hep-3B) through an increase of p21 expression, suggesting that p53 might not be involved in the magnolol-induced upregulation of p21 expression [1]. To completely map the signaling pathways involved in the magnolol-induced upregulation of p21 expression, more experiments need to be done.

In summary, we demonstrate for the first time that magnolol might activate the CaM/Ras/Raf-1 pathway to induce ERK activation, which in turn increases p21 levels, and finally causes cell-cycle arrest at the G_0/G_1 phase. Based on the results from the present study and our previous study, we propose

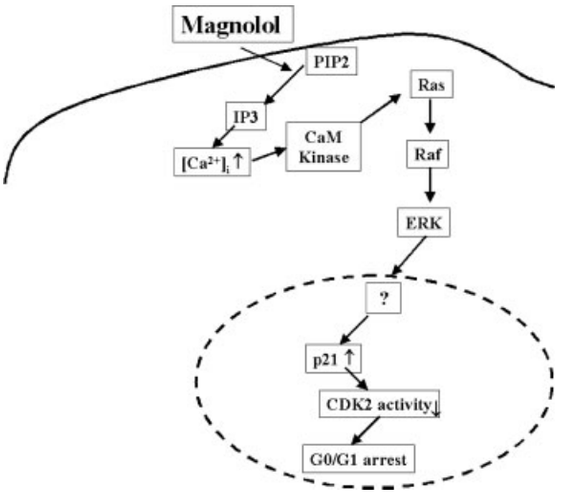

Figure 8. Proposed signaling pathway leading to p21 upregulation by magnolol-induced ERK activation via Ras/Raf-mediated pathway.

a model of the molecular mechanisms of magnolol-induced cell-cycle arrest in COLO-205 as shown in Figure 8. In the present study, we also demonstrated that transcriptional regulation is involved in the magnolol-increased the p21 protein levels (Figure 1). Although further experiments need to be done in order to delineate the transcriptional factor(s) and signal transduction pathways involved in the ERK-mediated upregulation of p21 mRNA and protein, the findings from the present studies suggest that magnolol activates the CaM/Ras/Raf-1/ERK pathway to induce the inhibition of proliferation in the magnolol-sensitive cancer cells and thus might provide a novel strategy for cancer therapy.

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