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American Ginseng Inhibits Induced COX-2 and NFKB Activation in Breast Cancer Cells

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Background. **Epidemiologic evidence suggests reduced breast cancer mortality in users of American Ginseng (AG) (*Panax quinquefolium*). We hypothesized that AG extract decreases proliferation of human breast cancer cells via an anti-inflammatory effect applicable to the prevention of breast and other cancers.**

Material and Methods. **A defined lyophilized aqueous extract of AG (LEAG) was dissolved in DMSO 1mg/mL, and serially diluted in saline. The cell lines MDA MB 231 and MCF7 were stimulated with the phorbol ester PDBu and treated with 100–500 mcg/mL LEAG. Proliferation was measured by MDA assay. Induced COX-2 expression was assayed by ELISA. Activation of NFκB by phosphorylation of the p65 subunit was quantified by CASE (cellular activation of signaling ELISA).**

Results. **Both cell lines had reduced proliferation when treated with LEAG. PDBu stimulation of MDA MB 231 increased expression of the COX-2 protein 20-fold at 48hours ($P < 0.005$). COX-2 protein expression remained at baseline concentrations in PDBu-treated MDA MB 231 cells exposed to100 mcg/mL LEAG. The CASE assay showed a 4-fold increase in p65 activation 24hours after PDBu treatment in normal medium, while phosphorylated p65 dropped below baseline in the cells treated with PDBu plus LEAG.**

Conclusion. **In MDA MB 231, COX-2 was inducible with PDBu. This induced COX-2 expression was blocked by 100 microgram/mL LEAG in a time course consistent with the decline in the activated p65 subunit of NFκB. These results provide an anti-inflammatory mechanism for a possible anti-cancer effect of American Ginseng.**

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Key Words: **breast cancer; ginseng; COX-2; NFκB; prevention; inflammation.**

INTRODUCTION

The availability of long-term maintenance therapy is one factor that allows for a favorable overall survival for-hormone-responsive breast cancer. Tumors that do not express estrogen or progesterone receptors rely on other cell signaling pathways to drive proliferation and resistance to apoptosis. The inducible form of cyclo-oyxgenase, COX-2, has been found in to majority of invasive breast cancers as well as ductal carcinoma in situ (reviewed in [1]). COX-2 over-expression has been implicated in the progression of cancer by increasing proliferation, invasiveness and angiogenesis. COX-2 up-regulation may also occur in hyperglycemia. Human endothelial cells cultured in high glucose conditions show increased COX-2 mRNA and protein and increases in oxidative stress [2]. A number of epidemiologic studies have compared breast cancer rates among subjects with diabetes versus those with normoglycemia. Retrospective studies show a mild positive correlation (RR 1.2-2.0), but researchers admit that confounding variables tend to weaken the association [3]. We have been studying a possible connection between the epidemiological evidence of an anticancer effect of ginseng and the known and potential systemic effects of ginseng.

American Ginseng (AG) (Panax quinquefolium) is an herb that is widely taken for its purported tonic effects. Epidemiologic evidence such as the Shanghai Breast Cancer Study [4] suggests a reduced mortality risk from breast cancer among ginseng users compared to never-users, however, the variable composition and possible estrogenicity of ginseng supplements prohibit

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recommendation to breast cancer patients. Studies from this and other laboratories have shown that American ginseng and purified components, ginsenosides, may inhibit cancer cell proliferation by inducing gene and protein expression of the cell cycle regulatory protein p21, thus arresting tumor cell cycle progression [5], by inducing cancer cell apoptosis through activation of caspase-3 protease via a bcl-2-insensitive pathway and by sensitizing multidrug-resistant tumor cells to chemotherapy [6, 7]. We previously demonstrated that our defined aqueous extract of AG is nonestrogenic and can inhibit breast cancer cell proliferation [8].

Ginseng and ginsenosides also have metabolic effects which may reduce the risk of cancer thought to be associated with the inflammatory and hyperinsulinemic state known as the metabolic syndrome [9] American ginseng in doses of 1 to 3 g taken 40 min before a glucose challenge test reduced postprandial glycemia in both diabetic and nondiabetic subjects [10, 11]. A reduction of insulin resistance in 3T3-L1 adipocytes treated with ginsenoside Re was mediated by down-regulation of NFκB activation [12].

The promoter region of the COX-2 gene contains a binding site for the p65 subunit of NFκB-3, and therefore signals that activate NFκB can be shown to induce COX-2 expression [13, 14]. These correlations imply that the anti-hyperglycemic effect of ginseng may be concomitant with a down-regulation of COX-2 expression.

Our hypothesis is that American Ginseng extract decreases proliferation of human breast cancer cells via an anti-inflammatory effect that may be applicable to the prevention of breast and other cancers. This hypothesis was tested by a three-pronged approach to evaluate the effect of LEAG on proliferation, induction of COX-2, and the upstream signal for COX-2 induction, NFκB activation. The effect of American Ginseng extract was studied in cultured breast cancer cell lines by immunohistochemical (IHC) stains for cyclin D1, Ki67, and COX-2 to assess the feasibility of using IHC of these proteins as biomarkers of therapeutic response to American Ginseng extract in a clinical trial.

MATERIAL AND METHODS

Reagents

The following antibodies were obtained from Santa Cruz Biotechnology Inc. (www.scbt.com): COX-2 clone M-19, Ki-67 clone H-300, and cyclin D1 clone DCS-6. These are either in established clinical use (Ki-67) or have been published as equal or better specificity to Western blot [15].

Lyophilized Extract of American Ginseng (LEAG)

American ginseng root, *(Panax quinquefolium* L.) was supplied by the Wisconsin Ginseng Board. The extraction process and active constituents have been previously described [16]. Certification of the product was performed by ConsumerLabs, LLC (White Plains, NY). LEAG was dissolved in 1 mg/mL DMSO stock solution and further diluted with phosphate buffered 0.9% sodium chloride for concentrations of 5–500 mcg/mL. Cell lines: Human breast cancer cell lines MCF-7, T47D, SK BR 3, and MDA-MB-231 were obtained from American Type Culture Collection (Manassas, VA), and maintained in a normal medium consisting of DMEM supplemented with 10% fetal bovine serum, 200 mM glutamine and penicillin. *In vitro* proliferation assays: Cells were plated 5000 cells/well into 96-well plates and incubated overnight at 37°C and 5% CO_2 in normal media before challenge with LEAG 5–500 mcg/mL for 24-and 48-h exposures. MTS substrate (Cell Titre96 Aqueous Non-radioactive Cell Proliferation Assay; Promega Corp., Madison, WI) was then added, plates were incubated for 3 h and spectrometry was performed to read absorbance at 490 nm. Proliferation was graphed as percent of normal control medium. Assays were run in triplicate and repeated in two separate experiments.

INDUCED COX-2 and Phospho-ERK(1/2) ELISA

The cell lines MDA MB 231 and MCF7 were stimulated with 1 μM phorbol 12,13-dibutyrate (PDBu), a protein kinase C (PKC) activator known to induce cyclo-oxygenase-2 (COX-2) expression. These preparations were then incubated in control medium and 50, 100, 250, and 500 mcg/mL aqueous extract of LEAG. Induced COX-2 expression was quantified by the TiterZyme EIA human COX-2 Enzyme Immunometric Assay kit (Assay Designs, Ann Arbor, MI, cat. no 900-094) and p-ERK by Titerzyme EIA Phospho-ERK (1/2) (Assay Designs, cat. No 900-098) according to the manufacturer's protocol. Experiments were performed twice, each time with triplicate wells and results expressed as the mean.

Histology

Cells were grown on sterile chambered slides (LAB-TECH) with normal medium, 1 μM PDBu alone (positive control), and PDBu in combination with LEAG (100, 250, and 500 mcg/mL) for 24 and 48 hs. The cells were fixed in 4% methanol-free formaldehyde, pH7.4 for 25 min at 4°C and then washed twice for 5 min in PBS at room temperature. After incubation with primary antibody, the slides were processed according to the manufacturer's protocol using the R.T.U Vectastain Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA, cat. no. PK-7200).

Cellular Activation of Signaling ELISA (CASE)

The effect of various concentrations of LEAG on NFκB pathway activation in MDA MB-231 cells stimulated with PDBu was measured by the method of CASE (SABiosciences, Frederick, MD). Activation of NFκB is quantified by the phosphorylation of the p65 subunit. Briefly, cells were plated 10^4 cells/well into 96-well plates and incubated overnight to reach 50% confluence before challenge with LEAG alone or PDBu ± LEAG 100, 250, or 500 mcg/mL for 24- and 48-h exposures. Cells were fixed to the wells with formaldehyde and processed for quenching of endogenous peroxidase, washed, and processed for antigen retrieval by microwaving in buffer per manufacturer's protocol. Primary antibody to phospho-p65 or total p65 was added to triplicate wells. Negative control wells were processed without primary antibody. Visualization with secondary antibody and developer solution was performed and read on an ELISA plate reader at 450 nm. The plates were then rinsed, air-dried, treated with cell staining buffer, and read at 595 nm. The quantity of phospho-p65 and total p65 was normalized to the relative cell number by the ratio of OD_{450} to OD_{595} for each well. The percent activation of NFκB was calculated as the ratio of phospho-p65 to total p65 for the same experimental condition. Experiments were performed twice, each time in triplicate wells and the results expressed as the mean.

RESULTS

All cell lines had reduced proliferation when treated with LEAG, with MDA MB 231 (IC50 250 mcg/mL) and SK BR 3 (IC50 of 50 mcg/mL) showing greater susceptibility than MCF7 (IC50 1000 mcg/mL) (Fig.1). Immunohistochemical staining of cells grown in normal medium ± 100–500 mcg/mL LEAG showed decreased expression of the proliferation markers Ki-67 and cyclin D1 (Fig. 2).

Only MDA MB 231 cells had detectable basal levels of COX-2 by ELISA. Basal COX-2 expression in MDA MB 231 cells was reduced in a dose-dependent fashion to LEAG alone at 100–500 mcg/mL (Fig 3A). There was no induced COX-2 protein expression in MCF7 cells treated with PDBu or PDBu plus LEAG (Fig 3B.) When the MDA MB 231 cell line was treated with PDBu, the expression of the COX-2 protein increased 20-fold at 48 h ($P < 0.005$). Induced COX-2 expression was abrogated in cells exposed to 100 mcg/mL LEAG (Fig 3C). COX-2 immunohistochemical staining of MDA MB 231 cells grown in medium with and without LEAG showed that AG eliminated COX-2 expression (Fig 4).

Phospho-ERK levels were high 24 h after plating for both MCF-7 and MDA MB 231 cells. In MCF-7, PDBu decreased rather than increased p-ERK, and LEAG + PDBu further decreased p-ERK at 48 h (Fig. 5A). MDA MD 231 cells showed no response of p-ERK levels to either PDBu or PDBu + LEAG (Fig. 5B).

The CASE assay performed on MDA MB 231 cells showed that when cultured in normal medium + LEAG 100 mcg/mL, total p65 increased over 48 h, but the activated phospho-p65 decreased nearly to zero (Fig. 6A). A PDBu challenge resulted in a 4-fold increase in phospho-p65, reaching 80% of total p65 protein by 24 h. The percent of phospho-p65 in cells treated with PDBu plus LEAG 100–500 mcg/mL decreased in a dose-dependent manner from 10%–40% of total p65 protein at 2 h to 0%–20% of total protein at 24 h (Fig. 6B).

DISCUSSION

The four cell lines used in the proliferation assays comprise different properties in breast cancer. T47D has properties of a low-grade tumor and expresses ER, PR, E-cadherin, and MCF-7, and has a low invasive capability and expresses ER, PR, E-cadherin, and epidermal growth factor receptor. Our previous study on the mechanism of LEAG in the reduction in proliferation of MCF-7 has proposed a dose-dependent decrease in phospho-MEK1/2 and -ERK1/2 and

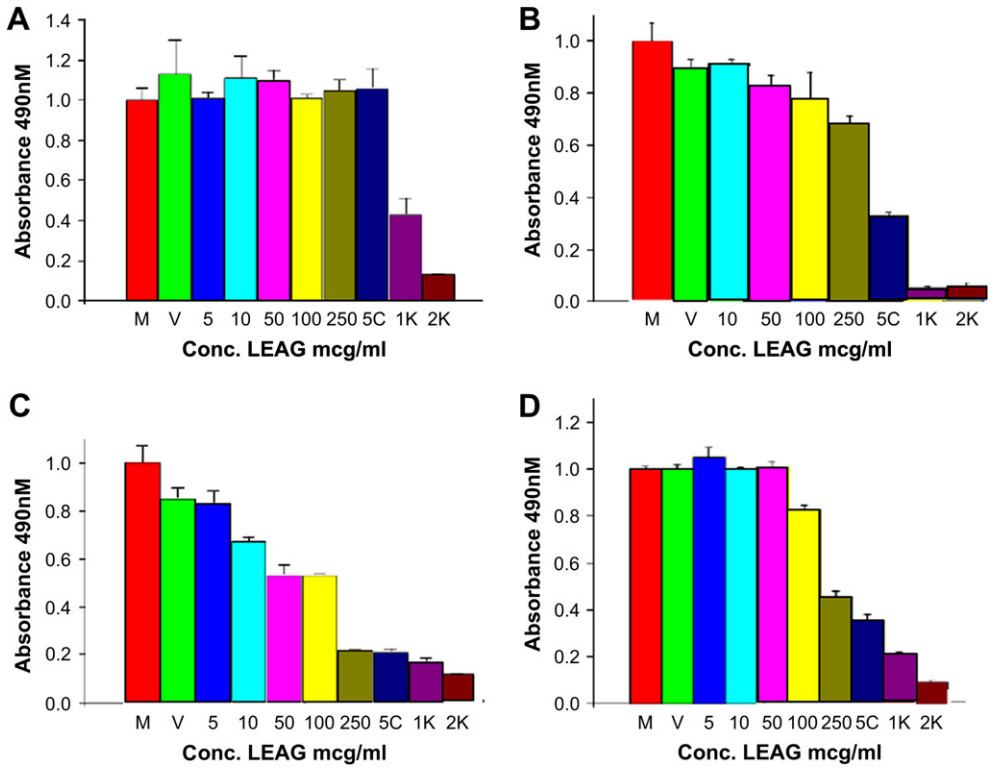

FIG. 1. Dose-response of a panel of ER+ and ER– breast cancer cell lines to LEAG at 24 hours. (A) MCF-7; (B) T47-D; (C) SKBr-3; (4) MDB MB 231. LEAG = lyophilized extract of American ginseng; M = media alone; V = media with vehicle; 5C = 500; 1 K = 1000; 2 K = 2000. (Color version of figure is available online.)

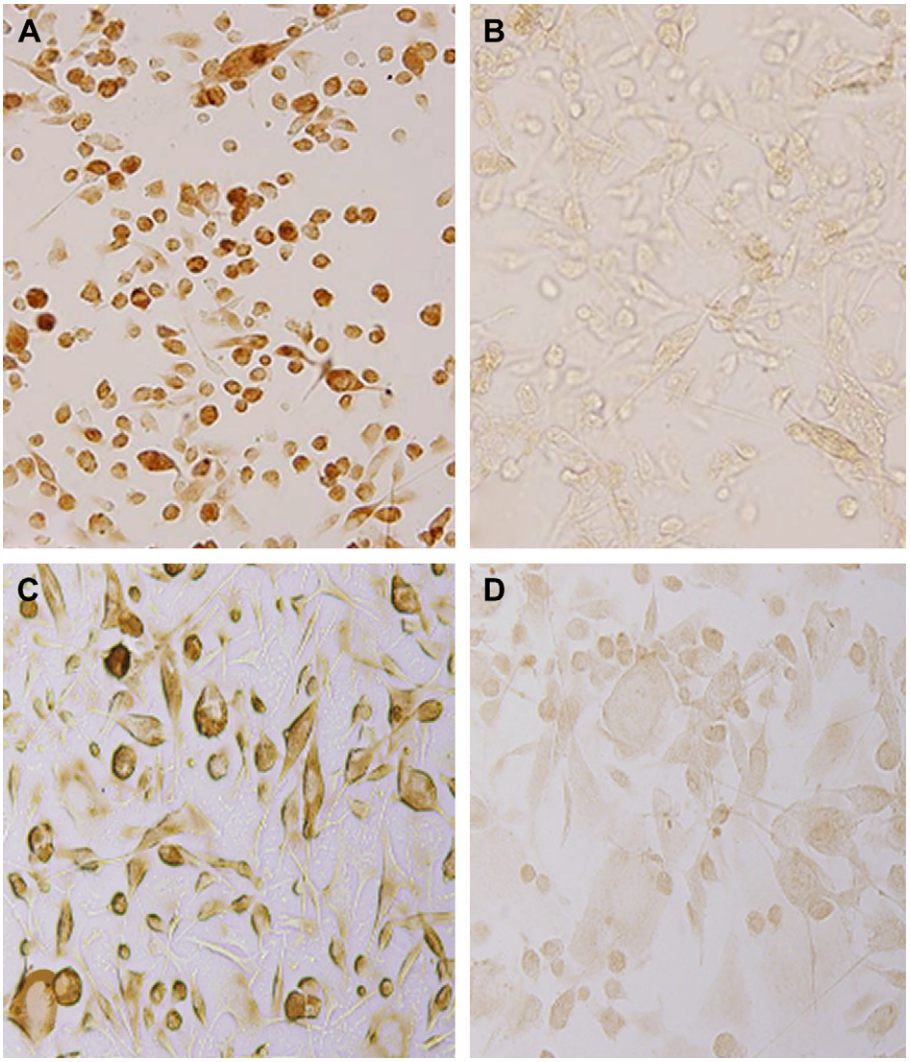

FIG. 2. Effect of LEAG on proliferation markers. MDA MB 231 cells were cultured on chambered slides with LEAG 100–500 mcg/mL and immunohistochemically stained. (A) Cyclin D-1, normal medium; (B) cyclin D-1, LEAG 500 mcg/mL; (C) Ki67 normal medium; (D) Ki-67, LEAG 100 mcg/mL (×200). LEAG = lyophilized extract of American ginseng. (Color version of figure is available online.)

increase in Raf-1 kinase inhibitor protein (RKIP), thereby reducing growth and survival signals through the MAPK system [8]. Our current finding that p-ERK decreased at 48 h with PDBu + LEAG treatment in MCF-7 was consistent with this, although this was not seen in MDA MB 231.

The MDA MB231 cell line has an invasive phenotype with metastatic properties. This cell line has lost the expression of ER, PR, and E-cadherin receptors, and was found in this study to have high baseline and inducible COX-2 expression. Since COX-2 induction was abrogated by LEAG, and LEAG had no effect on p-ERK in MDA MB 231, we looked further down the signal cascade to activation of NFκB, which was reduced in a dose and time response consistent with inhibition of induced COX-2 expression.

SK BR 3, a phenotypically a poorly-differentiated adenocarcinoma, expresses mutant ERα, the putative estrogen receptor GPR30 and over-expresses the HER2/c-erb-2 receptor. An interesting finding in this study is that the more aggressive tumor phenotypes showed growth inhibition at lower concentrations of LEAG. SK BR 3 did not show measurable COX-2 induction by PDBu and was not further studied here, however, it has been previously reported that SK BR 3 is highly sensitive to COX-2 inhibitors. This seeming paradox may be explained by a previous report that the expression of COX-2 is inversely correlated to the levels of ErbB2 [17], but prostaglandin E2 (a product of COX-2 activity) strongly up-regulates aromatase activity and proliferation in SK BR 3 [18]. Therefore, SK BR 3 may be dependent on low levels of COX-2 expression for growth signaling and thus show potent growth inhibition with LEAG.

Our previous work showed that LEAG decreased proliferation of human breast cancer cells and sensitized

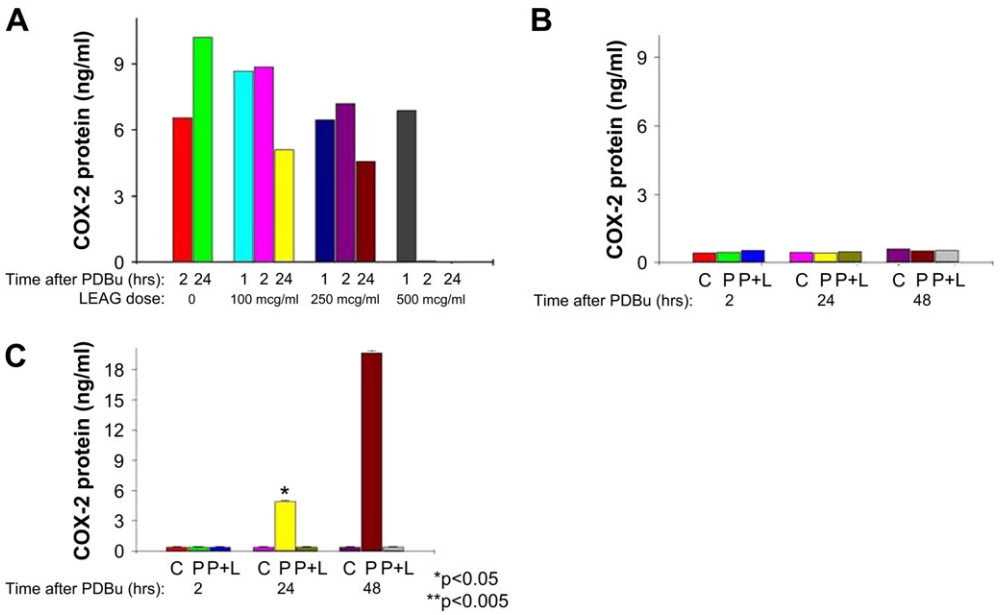

FIG. 3. LEAG reduces baseline and PDBu-induced COX-2 expression. (A) Dose response of MDA-MB-231 to LEAG; (B) MCF-7 shows no response of COX-2 expression to PDBu alone or with LEAG; (C) MDA MB 231 shows a 20-fold increase in COX-2 protein at 48 h after PDBu challenge. The response is abrogated in cells cultured with LEAG 100 mcg/mL at the time of PDBu. C = control (medium alone); PDBu (P) = 1 μM phorbol 12, 13-dibutyrate; LEAG = lyophilized extract of American ginseng; L = LEAG 100 mcg/mL. (Color version of figure is available online.)

them to the chemotherapy agent doxorubicin [19]. In the current study, a mechanism that could account both of these observations was explored by assaying the effect of LEAG on several of the activation and signaling sites of NFκB. NFκB exists in the cytoplasm in an inactive state that is maintained by binding with the protein, IκB. Multiple cell membrane signals such as the binding of growth factors, release of reactive oxygen species, and PKC activators including phorbol esters can activate pathways that converge on IκB kinase (IKK) to release IκB and allow p65 phosphorylation, leading to dimerization with the p50 subunit and translocation of the activated NFκB to the nucleus where it acts as a transcription factor to up-regulate a number of proteins involved in the growth and inflammatory response.

Both the proliferation markers Ki-67 and cyclin D1 were down-regulated by LEAG, in association with a dose-dependent decrease in cell proliferation. NFκB binds to a proximal binding site to activate transcription of the cyclin D1 promoter [20]; the down-regulation of NFkB by LEAG could thereby lead to cell cycle arrest. We found the effect to be on p65 phosphorylation at the level of IKK, downstream or independently of the ERK1/2 arm of the MAPK pathway, since p-ERK was unaffected by LEAG under the same conditions that abrogated phospho-p65 accumulation and COX-2 induction.

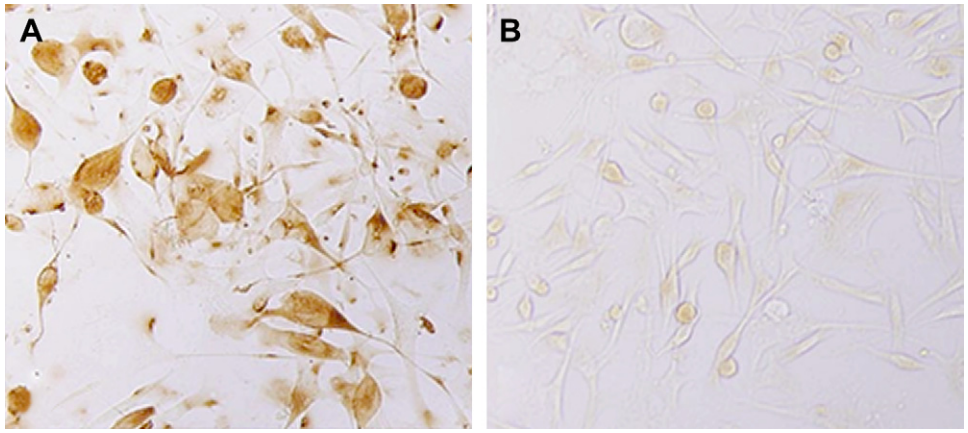

FIG. 4. Effect of LEAG on COX-2 protein expression. MDA MB 231 cells were cultured on chambered slides with LEAG and stained with COX-2 clone M-19 antibody (brown). (A) Normal medium; (B) LEAG 100 mcg/mL. ($\times$200). LEAG = lyophilized extract of American ginseng. (Color version of figure is available online.)

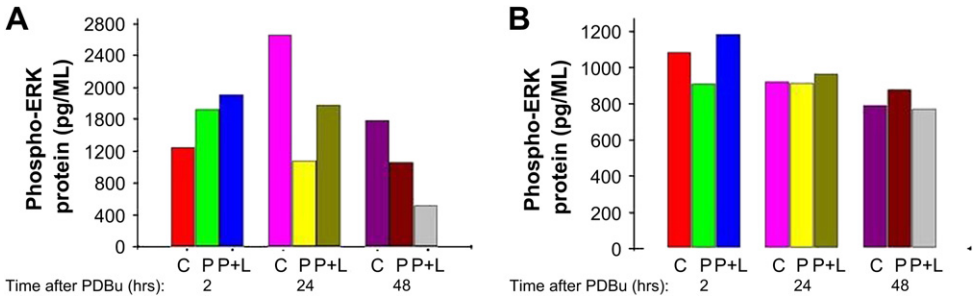

FIG. 5. ERK phosphorylation response to PDBu with and without LEAG. (A) MCF-7; (B) MDA-MB-231. Both cell lines had a high level of p-ERK 24 hours after plating in normal medium that was not increased by PDBu with or without LEAG. C = control (normal medium); ERK = extracellular receptor kinase; LEAG = lyophilized extract of American Ginseng; L = LEAG 100 mcg/mL; PDBU (P) = 1 μM phorbol 12, 13-dibutyrate. (Color version of figure is available online.)

NFκB also contributes to resistance to chemotherapy by up-regulating COX-2 and anti-apoptosis pathways. Interestingly, doxorubicin induces NFκB activation by activating IKK. By inhibiting NFκB activation at the level of IKK, LEAG could specifically oppose that mechanism of doxorubicin resistance.

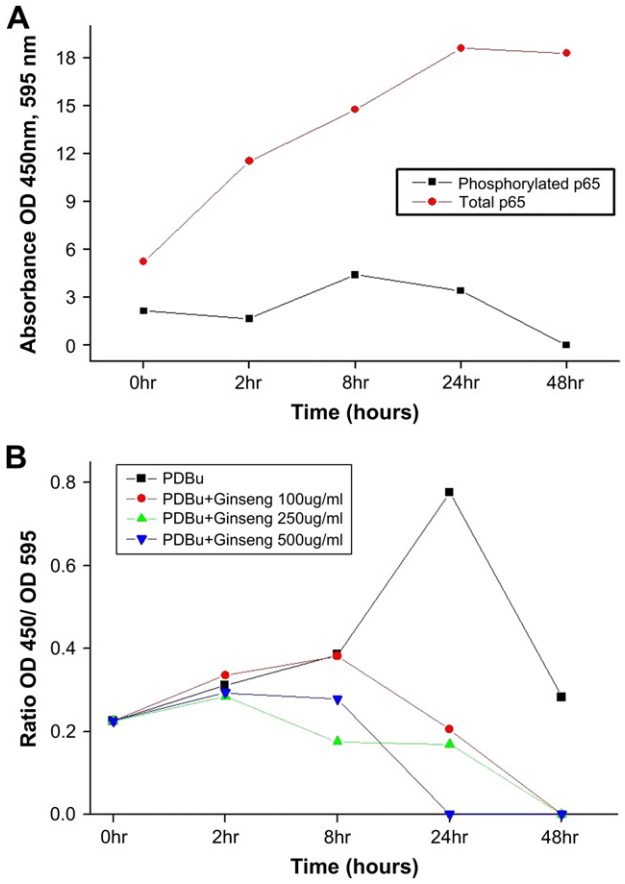

FIG. 6. Effect of LEAG on NFκB activation. A cell-based ELISA detecting phosphorylated NFκB p65 subunit was used as the endpoint of NFκB activation. (A) MDA MB 231 cells were cultured in normal medium plus LEAG 100 mcg/mL at time 0. Total p65 protein increased over time but phospho-p65 decreased. (B) Ratio of phospho-p65/total p65 protein in MDA MB 231 cells treated with 1 μM phorbol 12, 13-dibutyrate alone and with LEAG 100-500mcg/mL. A lower ratio indicates inhibition of NFκB activation. At 24 h, 80% of p65 is phosphorylated in the PDBu-alone cells compared with 0%–20% in the LEAG-treated cells. (Color version of figure is available online.)

In addition to the potential for treatment of breast cancer, there is a cancer prevention prospect for LEAG in its reduction of COX-2 expression. COX-2 is over-expressed in premalignant tissues, including breast. Visscher *et al.* analyzed 235 archived breast biopsies of atypical ductal hyperplasia for COX-2 expression by IHC and found that the risk of subsequent diagnosis of breast cancer increased in proportion to the COX-2 over-expression (RR 2.63 for 2+ expression; RR 5.66 for 3+ expression) [21]. Although inhibition of COX-2 has been a target of interest in the prevention of colon and breast cancer, initial trials for COX-2 inhibitors, such as the arthritis drug celecoxib in the treatment or prevention of breast cancer, were halted by evidence of cardiotoxicity [22]. Ginseng is an herbal supplement that has been consumed by humans for thousands of years. Remarkably little documentation exists for any toxic effects of ginseng, or of the clinically-achievable serum levels of the active components of enterally-administered ginseng. We are addressing these questions under FDA supervision in a phase II clinical trial using this particular defined extract of American ginseng (http://clinicaltrials.gov/ct2/show/NCT00631852).

In conclusion, in MDA MB 231, COX-2 was inducible with PDBu. This induced COX-2 expression was blocked by 100 mcg/mL LEAG in a time course consistent with the decline in the activated p65 subunit of NFκB. These results provide an anti-inflammatory mechanism for a possible anticancer effect of American ginseng. The observation that the ER-negative cell line is susceptible is encouraging because there is presently no long-term maintenance therapy for ER-negative breast cancer. A clinical trial to examine the effects of LEAG on normal and malignant breast tissue is in progress.

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