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Original article

Regulation of inducible nitric oxide synthase by dietary phytoestrogen in MCF-7 human mammary cancer cells

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Abstract — We examined the effects of the phytoestrogen biochanin A on the growth of the MCF-7 human breast cancer cell line. The results showed that biochanin A treatment induced dose- and time-dependent inhibition on MCF-7 cell growth at concentrations above 20 $\mu g \cdot mL^{-1}$. An examination of treated MCF-7 cell morphology revealed condensation of the chromosome and dehydration of the cytoplasm, suggesting apoptosis as an important factor in biochanin A-related cell growth inhibition. The results also showed that at a concentration of 40 $\mu g \cdot mL^{-1}$, biochanin A decreased the levels of inducible nitric oxide synthase, thus inhibiting the production of nitric oxide, a known second messenger and inducer of apoptosis, and affecting the overall cell protein pattern. No significant difference in superoxide dismutase protein levels were, however detected at concentrations of 40 or 100 $\mu g \cdot mL^{-1}$ of biochanin A. The data suggest that the inhibitory effects of biochanin A on human breast cancer cell growth are linked to inducible nitric oxide synthase and the associated production of nitric oxide.

phytoestrogen / nitric oxide synthase / superoxide dismutase / proliferation

Résumé — **Régulation de la NO-synthase inductible dans des cellules cancéreuses mammaires humaines MCF-7 par des phyto-œstrogènes alimentaires.** Les effets d'un phyto-œstrogène, la biochanine A, sur la croissance d'une lignée cellulaire MCF-7 humaine de cancer de sein, ont été étudiés. Les résultats montrent qu'un traitement à la biochanine A a induit une inhibition dose et temps dépendante de la croissance des cellules MCF-7 pour des concentrations supérieures à 20 $\mu g \cdot mL^{-1}$. L'examen morphologique de cellules MCF-7 traitées révèle une condensation chromosomale et une déshydratation du cytoplasme, suggérant que l'apoptose est une facteur important de l'inhibition de croissance due à la biochanine A. A des concentrations supérieures à 40 $\mu g \cdot mL^{-1}$, la biochanine A

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diminue les taux de monoxyde d'azote synthase inductible, inhibant ainsi la production de monoxyde d'azote, second messager induisant l'apoptose et modifiant les protéines cellulaires. Cependant, aucune différence significative n'a été observée pour les taux de protéine de la superoxyde dismutase à des concentrations de 40 ou de 100 $\mu g \cdot mL^{-1}$ de biochanine A. Les résultats suggèrent que l'effet inhibiteur de la biochanine A sur la croissance des cellules cancéreuses humaines du sein est lié à la monoxyde d'azote synthase inductible et à la production associée de monoxyde d'azote.

phyto-œstrogène / monoxyde d'azote synthase / superoxyde dismutase / prolifération

1. INTRODUCTION

Phytoestrogens are a family of plant phenolics found in soybeans, whole-grain products, a variety of seeds, and many other legumes [3]. Their estrogenic nature is due to their structural similarity with 17 β-estradiol, the principal physiological estrogen found in animals [19, 21]. Epidemiological data suggest that a diet rich in phytoestrogens provides protection against several forms of cancer, particularly those that are hormone-dependent [1]. Soybean-based diets have been shown to decrease the risk of mammary tumours in rats induced by radiation [29]. Other studies point to a lower risk of breast and prostate cancers in populations with higher levels of phytoestrogen excretion [12]. With steroidal estrogens, however, plant estrogens have the potential to exert adverse as well as beneficial influences, including permanent infertility, liver malfunction, and hormone-dependent diseases [4, 16]. These conflicting outcomes make it important to fully investigate the effects and mechanisms of phytoestrogens.

Daidzein and its precursor, biochanin A, which are two of the more important phytoestrogens, are found in substantial concentrations (5% dry mass or less) in two major pasture legumes *Trifolium subterraneum* (subterranean clover), and *T. pratense* (red clover) as well as in soybeans (up to 100–300 $mg \cdot 100\ g^{-1}$) [6]. Previous studies on the estrogenic-like activity of biochanin A and daidzein have shown that they inhibit MCF-7 and T-47D human breast cancer cell proliferation, most likely via the apoptosis pathway, an active process of cellular self-destruction with unique defining morphological and molecular characteristics [8, 10, 28]. Nitric oxide (NO) has been identified as one of the signals which triggers or interferes with execution steps of apoptosis depending on the cell types [17, 23]. A number of diseases and disorders are directly related to NO overproduction or underproduction [2, 7].

In mammalian tissues, NO is synthesized from the guanido nitrogen group of L-arginine and molecular O2 by NO synthase (NOS) isoforms, including inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS) [22]. Miller et al. [20] reported that estrogen and progesterone inhibit iNOS gene expression and NO production. In addition, estrogens inhibit the induction of iNOS protein and NO release in interferon-treated macrophages; these effects can be blocked by treatment with antiestrogen, tamoxifen and ICI 182,780 [9]. It has also been reported that NO release and iNOS activity is enhanced by estrogen in the uterine arteries of non-pregnant sheep [31]. The induced production of iNOS was also observed in bovine endothelial cells and human microglial cells by the basic fibroblast growth factor [5, 13].

Recently, it has become increasingly evident that NO behaves as either a beneficial or harmful mediator depending on the presence of factors such as oxidative stress [21]. Accumulation of intracellular hydrogen

peroxide was suggested to cause hydroxyl radical-induced DNA base lesions in breast cancer tissues [26]. Such activity is lower in many tumor cells compared to the normal counterpart [24]. In some tumor cell lines, the induction and overexpression of the superoxide dismutase (SOD) gene whose gene product catalyzes the dismutation of superoxide (O_2^-) to oxygen and H_2O_2 led to the suppression of the malignant phenotype [18]. The SOD family of enzymes is characterized by the metals they contain, including the copper and zinc form of SOD (CuZnSOD), a soluble enzyme found in the cytoplasm of all mammalian cells, and manganese SOD (MnSOD), a mitochondrial enzyme [14]. In cultured rat granulosa cells, CuZnSOD has been shown to regulate the activity of aromatase cytochrome P450 which catalyzes the conversion of androgens to estrogens in ovaries [15]. When the ovaries are removed there is a marked decreased in CuZnSOD activity, suggesting that such an activity is modulated by estrogen [11].

In this study, we examined whether the estrogenic activity of biochanin A is associated with iNOS and SOD-regulated levels of free radicals in human breast cancer cells. The results from measuring iNOS and SOD levels in response to biochanin A treatment showed that the iNOS protein may be involved in the inhibition of human breast cancer cell growth.

2. MATERIALS AND METHODS

2.1. Chemicals

Biochanin A was purchased from the Sigma Chemical Co. (St.-Louis, MO) and the MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The phenol red-free Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and the antibiotic/antimycotic mix used in this study were purchased from GIBCO BRL (Gaithersburg, MD). The biochanin A solution was prepared in dimethylsulphoxide (DMSO) and stored at –20 °C.

2.2. Cell culture conditions

MCF-7 cells were grown in phenol red-free DMEM supplemented with either 10% FBS or 3 × dextran/charcoal-stripped FBS. The dextran/charcoal stripped FBS was prepared by passing the FBS through dextran coated charcoal three time at 4 °C and then filter sterilized to deplete the steroids including estrogens from the FBS. The cultures were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2 and fed every 2 days.

2.3. Cell proliferation assays

The cells were cultured until 80–90% confluence was attained and the medium was then changed to serum-free DMEM for an additional 24 h to synchronize the cells. The number of collected cells per milliliter was determined using the trypan blue dye exclusion method. The cells were seeded in 96-well plates in phenol red-free DMEM containing 10% 3 × dextran/charcoal-stripped FBS at 10^4 per well for the experiments. Following a 24 h pre-culture period to ensure attachment, the medium was removed and replaced with fresh phenol red-free medium supplemented with 10% dextran/charcoal-stripped FBS alone or with biochanin A. DMSO at equal dilutions was added in parallel control cultures, with final concentrations kept below 1% (v/v), causing no measurable effects on cell growth or cell morphology.

Following incubation, the WST-1 reagent was added into each well to determine the number of viable cells in each well according to the manufacturer's guidelines. The technique is based on the observed cleavage

of tetrazolium salt WST-1, leading to the formation of formazan dye by the succinate-tetrazolium reductase system, which belongs to the respiratory chain of the mitochondria and is active only in viable cells. The amount of formazan dye which results is directly correlated to the number of metabolically active cells in the culture. Formazan dye formation leads to an increase in the absorbance at the wavelength of 420 nm, as measured using a scanning multiwell spectrophotometer (ELISA reader). The difference between absorbances at 20 nm and at 650 nm representing the background was used as an index of viable cells in each well.

2.4. Cell morphology

An aliquot of 5×10^5 synchronized cells was seeded in each well of 6-well culture plates. After 24 h, biochanin A was added to each well to 100 $\mu g \cdot mL^{-1}$ and the cultures allowed to grow for an additional 4 days. Detached cells floating in the medium were collected, washed twice with PBS, and fixed in 75% ethanol by incubation at –20 °C for 20 min. Following centrifugation and removal of the supernatant, the cell pellets were washed once with PBS, incubated with PBS containing 0.1% Triton X-100 at room temperature for 20 min, then centrifuged again. After further removal of the supernatant, cell pellets were incubated with PBS containing 10 $\mu g \cdot mL^{-1}$ acridine orange at room temperature for 20 min, then washed twice with additional PBS. The cells were then resuspended in 0.1 mL PBS and applied to cover slides using cytospin (Kubota 5800) before being viewed and photographed with a Leitz Orthoplan microscope.

2.5. Immunoprecipitation and immunoblotting

Cell lysates of MCF-7 cells were prepared as described by Ying et al. [32]. Briefly, prepared cell lysates containing equal amounts of cellular protein after biochanin A treatment were immunoprecipitated overnight with 10 μL of anti-iNOS antibody at 4 °C. Next, 50 μL of protein A-agarose was added to the reaction mixture prior to incubation for another 12 h at 4 °C. Following centrifugation, precipitated immune complexes were solubilized and separated using 10% SDS-PAGE, then transferred to a nitrocellulose membrane. This membrane was incubated with anti-iNOS antibody. Other cell lysates were fractionated using 15% SDS-PAGE, western transferred and immunoblotted with anti-SOD antibody.

3. RESULTS AND DISCUSSION

Dietary phytoestrogens have been shown to influence proliferation in several cell lines, including mammary cancer cells. The results of this study showed that effects of biochanin A on MCF-7 cell proliferation were similar to those reported previously [10, 28]. As shown in Fig. 1, significant growth inhibition (IC_{50} of 25 $\mu g \cdot mL^{-1}$) was observed in cells incubated with biochanin A for 2 or 4 days. After 4 days, cell growth was stimulated when the biochanin A concentration fell below 10 $\mu g \cdot mL^{-1}$. This observed biphasic response of MCF-7 cells to biochanin A treatment was in agreement with those previously described [10].

Figure 2 represents data on the effects of biochanin A treatment on cell morphology. Most cells treated with 100 $\mu g \cdot mL^{-1}$ biochanin A for 4 days detached from the culture flask surface and floated in the medium. These floating cells were collected and stained with acridine orange (Fig. 2D). Compared to the staining in the control cells (Fig. 2B), the strong staining of the nuclei of biochanin A-treated cells suggested condensation of the nucleosome. Decreasing in the cytoplasmic volume was also noticed in the biochanin A treated cells. These morphological changes have been considered as indicators of apoptosis [30, 33] which

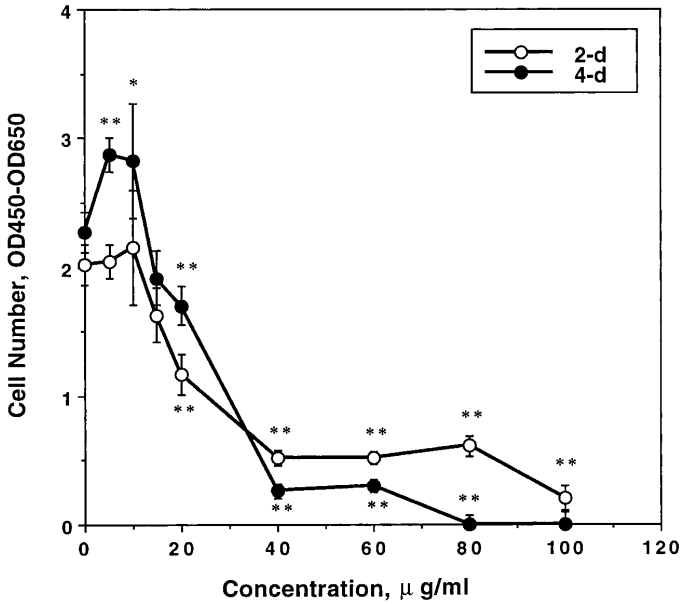

Figure 1. Concentration-dependent and time-dependent effects of biochanin A on MCF-7 cell proliferation. Cell numbers were determined following 2 or 4 days of growth in the presence of biochanin A at the indicated concentrations. The * and ** indicates significant ($P < 0.01$) and very significant ($P < 0.005$) differences compared to DMSO vehicle-treated control cells, respectively.

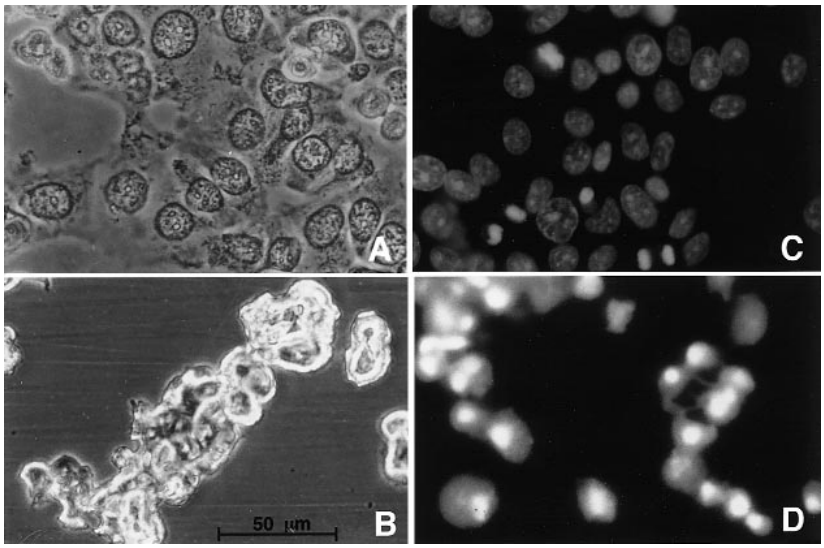

Figure 2. Changes in MCF-7 cell morphology following treatment with biochanin A. Cells were treated with 100 $\mu g \cdot mL^{-1}$ of biochanin A for 4 days and those cells which were detached from the culture flask surface and floated in the medium were collected and photographed directly (A) or stained with acridine orange prior to being photographed (B). DMSO-treated control cells were also photographed directly (C) or stained with acridine orange and photographed (D) for comparison.

may explain the death of cells treated with biochanin A. However, the possibility remains that the necrosis pathway is also involved in cell death.

Biochanin A treatment also affected cellular protein patterns in MCF-7 cells (Fig. 3A). The most noticeable change was the shift in protein distribution from a greater to lesser homogeneity. Following biochanin A treatment, proteins with higher molecular weights became the dominant species.

As shown in Figure 3B, biochanin A treatment at 40 $\mu g \cdot mL^{-1}$ led to a decrease of the iNOS protein level in MCF-7 cells. Immunoprecipitation of the cell lysates with anti-iNOS antibody was required prior to western blot analysis in order to visualize the iNOS signal. A previous report has also indicated that estrogen inhibited iNOS protein induction by means of an ER-mediated system [9]. This inhibitory effect noted in the present study may also be due to the ER protein present in the tested cells. On the contrary, the CuZnSOD protein level were not influenced by biochanin A treatment (Fig. 3C). SOD protein levels were similar in control cells and cells floating in culture medium following biochanin A treatment at 40 or 100 $\mu g \cdot mL^{-1}$, so it is doubtful that the observed inhibitory effects are associated with the SOD protein.

To further determine the potential effects of apoptosis, we examined DNA fragmentation in biochanin A-treated MCF-7 cells. Agarose gel electrophoresis failed to identify significant changes in the chromosomal DNA of treated cells (data not shown). This failure may be due to the low sensitivity of the detection method used, or it may be that biochanin A-induced apoptosis does not involve DNA fragmentation. Schulze-Osthoff et al. [25] showed that DNA fragmentation is not required for the induction of apoptotic cell death in certain signal pathways. Further examination of the formation of apoptotic bodies would facilitate a greater understanding of this mechanism.

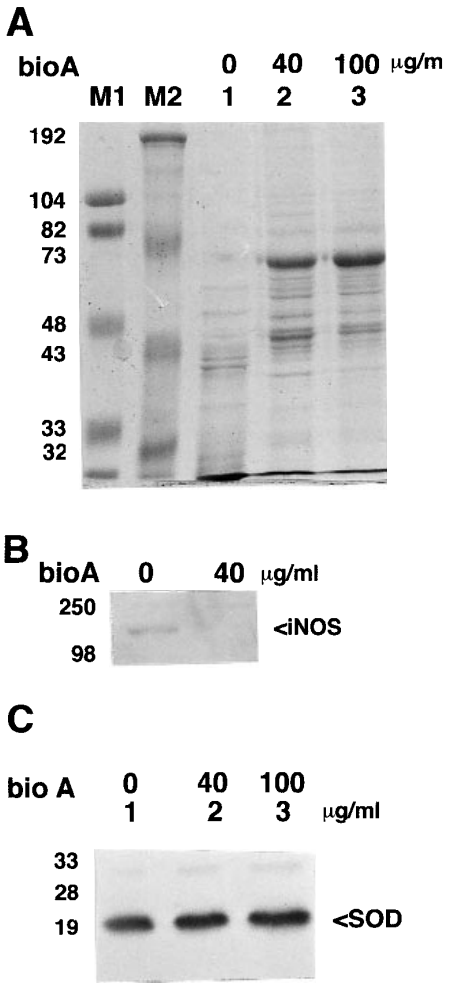

Figure 3. MCF-7 protein profiles and iNOS and SOD protein levels in response to biochanin A treatment. (A) Cell lysates prepared from those floating cells following biochanin A treatment at indicated concentrations for 4 days were fractionated by 15% SDS-PAGE gel electrophoresis and stained with Coomassie blue dye. M1 and M2: two sets of molecular weight markers in kDa. These cell lysates with equal amounts of protein were either (B) immunoprecipitated with anti-iNOS antibody followed by western blotting with anti-iNOS antibody or (C) western blotted with anti-SOD antibody. Cell lysates from MCF-7 cells treated in parallel with DMSO were designated as the zero concentration biochanin A. Molecular weight markers are indicated on the left in kDa.

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