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Effects of the dietary phytoestrogen biochanin A on cell growth in the mammary carcinoma cell line MCF-7

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Studies of the dietary phytoestrogen biochanin A on cell proliferation of the cultured estrogen responsive cells human breast carcinoma MCF-7 showed that biochanin A exhibits biphasic regulation on MCF-7 cells. At concentrations of less than 10 μg/mL, cells respond to biochanin A by increasing cell growth and de novo DNA synthesis. The addition of biochanin A at concentrations of greater than 30 μg/mL significantly inhibited cell growth and DNA synthesis in a dose-dependent fashion, resulting in an IC_{50} value of 40 μg/mL. The reversibility of these inhibitory effects by biochanin A appears also to be concentration dependent. Cells previously treated with high concentrations (>60 μg/mL) of biochanin A did not regain normal growth after treatment ceased. Biochanin A was cytostatic at low concentrations (<40 μg/mL) and cytotoxic at higher concentrations. Upon exposure to 100 μg/mL of biochanin A, cell morphology was severely altered, cell volume decreased, and condensation of cell components was clearly noticeable. In addition, biochanin A damaged cell membranes by increasing membrane permeability. These results suggest possible molecular and cellular mechanisms of the action of dietary phytoestrogens on estrogen target cells. (J. Nutr. Biochem. 10:510–517, 1999)

Keywords: legume; cell proliferation; nonsteroidal estrogen; biochanin A

Introduction

Detrimental substances present in forage for grazing ruminants have caused concern because of potentially deleterious effects on the animals. Phytoestrogenic substances that occurs in high concentrations (up to 5% dry matter (DM)) in clover, especially *Trifolium subterraneum* (subterranean clover) and closely related species and *T. pratense* (red clover), have been proposed to have an important influence on ruminant fertility.[1] Isoflavonoids are a group of natural plant substances that share structural similarity to natural animal estrogens, such as estradiol, and exhibit affinity for the estrogen receptor (ER).[2–4] These compounds have been considered to be responsible for the depression of fertility observed in sheep grazing in clover pasture.[5] Isoflavonoids have also been implicated in bovine infertility.[6]

The estrogenic activity of isoflavonoids has been investigated in biological assays such as the uterine weight increase of sheep or rat.[7,8] Clinical and epidemiologic data also indicate an association between the dietary consumption of soybeans, which contain large amounts of phytoestrogens, and protection from hormone-dependent disease in humans.[9] Veterinary studies suggest that these compounds may also be involved in hormone-dependent disease in sheep and captive cheetahs[10,11] and in developmental toxicity in rats and mice.[12,13] Reports on the effects of equol, a metabolite of formononetin responsible for reproductive disturbances in sheep and rats, are conflicting.[14,15] The meaning of these variable results is difficult to discern in the absence of more detailed cellular studies on the phytoestrogens.

We have undertaken a study of the cellular action of dietary estrogens by examining the estrogenic action of biochanin A, one of the major isoflavonoids in pasture legumes. With no appropriate ruminant system available,

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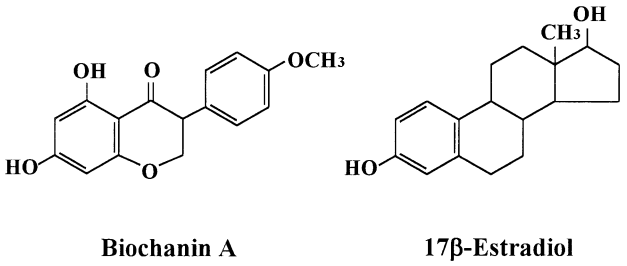

Biochanin A 17β-Estradiol

Figure 1 Structures of biochanin A and 17β-estradiol, the natural animal estrogen

we analyzed the estrogenic actions of biochanin A by using a cell line derived from human breast cancer cells (MCF-7) as the model system because most studies on the mechanism of phytoestrogen regulation have been performed in similar breast cancer cell systems.

Materials and Methods

Chemicals

Biochanin A (*Figure 1*) was purchased from Sigma Chemical Co. (St. Louis, MO USA). The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD USA). Phenol red-free Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and an antibiotic/antimycotic mix were purchased from GIBCO BRL (Gaithersburg, MD USA). A cell proliferation kit (WST-1), a 5-bromo-2′-deoxyuridine (BrdU) DNA synthesis labeling kit, and cell membrane permeability assay kit were purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). The solution of biochanin A was prepared in dimethylsulfoxide (DMSO) and stored at −20°C in the dark.

Cell culture conditions

The MCF-7 cells, which a human breast cancer cell line that contains mainly ER α (the classical ER), were grown in phenol red-free DMEM containing a 1X antibiotic/antimycotic mix, 5 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid, and 0.37% sodium bicarbonate.[16] The medium was supplemented with either 10% FBS or 3X dextran/charcoal-stripped FBS.[17] Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% carbon dioxide and fed every 2 days.

Proliferation assays

Cells were cultured in phenol red-free DMEM supplemented with 10% FBS until 80 to 90% confluence was reached and the medium was changed to DMEM without serum for an additional 24 hours to synchronize cells to the G_0/G_1 phase of the cell cycle. The cells were removed from the culture flasks with 0.05% trypsin and 3 mM EDTA in HBSS, collected by centrifugation at 500 × *g* for 3 minutes, and the number of cells per milliliter was determined by the trypan blue dye exclusion method. Cells were seeded in 96-well culture plates in phenol red-free DMEM containing 10% 3X dextran/charcoal-stripped FBS at 10,000 cells per well for the experiments. After a 24-hour preculture period to ensure attachment, the medium was removed and fresh phenol red-free medium supplemented with 10% dextran/charcoal-stripped FBS alone or with the preestablished concentrations of biochanin A was added. DMSO, at the same dilution, was added in parallel cultures as a control. Final concentrations of DMSO in the culture medium were kept below 1% (vol/vol), which caused no measurable effects on cell growth or cell morphology. At the end of incubation period, WST-1 reagent was added to determine the number of viable cells in each well according to the manufacturer's instructions. This technique is based on the fact that tetrazolium salt WST-1 is cleaved to form formazan dye by the succinate-tetrazolium reductase system, which belongs to the respiratory chain of the mitochondria and is active only in viable cells. Therefore, the amount of formazan dye formed is directly correlated to the number of metabolically active cells in the culture. The formation of formazan dye that exhibits absorbance at the wavelength of 450 nm was quantified using a scanning multiwell spectrophotometer [enzyme-linked immunosorbent assay (ELISA) reader; Emax precision microplate reader, Molecular Devices, Sunnyvale, CA USA]. The absorbance at A_{450} nm minus the absorbance at A_{650} nm, which represent cellular background, was directly correlated to the cell number. Each condition was represented in five separate wells per experiment and was repeated in at least four independent cultures.

DNA synthesis determination

The MCF-7 cells were grown, synchronized as described, and seeded to 96-well culture plates at the density of 5×10^4 cells per well in phenol red-free DMEM containing 10% dextran/charcoal-stripped FBS. After 24 hours, the medium was removed and fresh medium containing biochanin A or DMSO was added to each well. Cells were incubated for various periods of time and the amounts of de novo DNA synthesis in each well was measured by using the BrdU DNA synthesis labeling kit according to the manufacturer's instructions (Boehringer Mannheim Biochemicals). This assay is based on the detection of BrdU incorporated into the genomic DNA of proliferating cells. Cells were labeled by the addition of BrdU, which was incorporated in place of thymidine into the DNA of cycling cells. After removing the labeling medium, cells were fixed and the DNA was denatured. Anti-BrdU-POD antibody, a monoclonal antibody from mouse-mouse hybrid cells (clone BMG 6H6, Fab fragments) against BrdU conjugated with peroxidase (POD), was added and bound to the BrdU that was already incorporated into the newly-synthesized cellular DNA. The immune complexes were detected by the subsequent substrate reaction by the addition of tetramethylbenzidine and the reaction product was quantified by measuring the absorbance at A_{450} using an ELISA reader. Experiments were repeated at least twice and each condition was represented in four separate wells per experiment.

Cell morphology

Cells were cultured in DMEM containing 10% FBS and synchronized as described above. An aliquot of 5×10^5 cells was seeded into each well of six-well culture plates. After 24 hours of growth, biochanin A was added to each well to 100 μg/mL for an additional 4 days. Cells that remained attached to the plate surface were then washed with phosphate buffered saline (PBS) twice and photographed with a Leitz Orthoplan microscope (Wetzlar, Germany). Cells that became detached and floated in the medium were recovered by centrifugation, washed with PBS, and applied to cover slides using cytospin (Kubota 5800) to allow reattachment to the slide surface before they were viewed and photographed.

Cell membrane permeability assay

Synchronized MCF-7 cells were grown and treated with biochanin A as described. The culture supernatant was collected cell-free and incubated with the substrate mixture. The lactate dehydrogenase (LDH) activity in the supernatant was determined with a coupled

enzymatic reaction whereby the tetrazolium salt was reduced to formazan dye that was measured by an ELISA reader.

Statistical analysis

Data were analyzed by the method of least-squares analysis of variance using the GLM procedures[18] for a completely randomized design with biochanin A concentration as the treatment factor.[19] Treatment means were compared by the orthogonal contrast with the comparisons of each individual biochanin A concentration versus control blank.

Results

Biochanin A effects on MCF-7 cell proliferation

To study the kinetics of inhibition in cell proliferation, cell cultures were exposed to 0.1 to 100 μg/mL of biochanin A in medium containing 10% charcoal stripped FBS for 1 to 4 days (*Figure 2*). Biochanin A produced a reproducible biphasic response in the cell proliferation of MCF-7 cells. At concentrations of less than 10 μg/mL, cell proliferation was stimulated by biochanin A (*Figure 2A*). Cell proliferation was significantly enhanced ($P < 0.0001$) in MCF-7 cells treated with 1 μg/mL of biochanin A to approximately 123% that of controls. At the lowest concentration tested (0.1 μg/mL), biochanin A induced a 19% growth increase ($P < 0.005$) compared with DMSO-treated control cells. When concentrations were higher than 30 μg/mL, biochanin A exhibited inhibitory activity in the cell proliferation of MCF-7 cells in a dose-dependent fashion (*Figure 2A*). The MCF-7 cell proliferation was inhibited approximately 50% at 40 μg/mL and 78% at 100 μg/mL, which was the highest concentration of biochanin A tested. The inhibitory effects of biochanin on MCF-7 cell proliferation were clearly observed after the first day of administration (*Figure 2B*). No further noticeable inhibition by biochanin A on cell proliferation was detected following an additional incubation period of 4 days.

Biochanin A altered DNA synthesis in MCF-7 cells

The effects of biochanin A on cell proliferation may have been caused by regulating the cell cycle progression at the G_1-S phase. Therefore we examined the de novo DNA synthesis that occurred only during the S phase in MCF-7 cells treated with biochanin A (*Figure 3*). Biochanin A significantly influenced DNA synthesis at all concentrations tested (*Figure 3A*). Biochanin A exhibited a similar biphasic response in DNA synthesis as observed in the cell proliferation described above. DNA synthesis increased after treatment of biochanin A at low concentrations. An increase of 280% in DNA synthesis was observed at a concentration of 5 μg/mL. At 20 μg/mL, DNA synthesis in MCF-7 cells declined to 47% after being treated with biochanin A for 4 days. At 40 μg/mL, only residual DNA synthesis (4%) remained after exposure for 4 days. At concentrations greater than 40 μg/mL, no measurable DNA synthesis was present when cells were treated with biochanin A. An analysis of the changes in DNA synthesis from day 1 of biochanin A treatment in conjunction with the kinetics of biochanin A inhibitory effects on DNA synthesis revealed a correlation to cell proliferation (*Figure 3B*). DNA synthesis

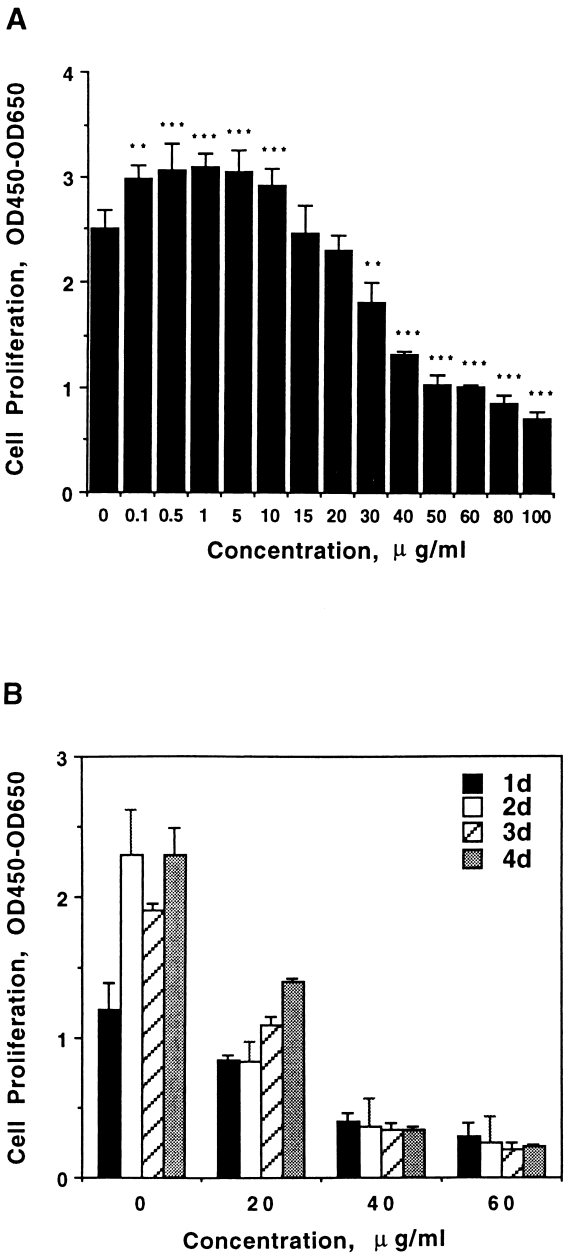

Figure 2 Effects of biochanin A on the proliferation of MCF-7 cells. *(A)* MCF-7 cells were treated with biochanin A for 2 days at the concentrations indicated or *(B)* for 0 to 4 days at preestablished concentrations. The values (mean ± SD) with ** and *** indicate significant difference ($P < 0.005$) and very significant difference ($P < 0.0001$), respectively, compared with the dimethylsulfoxide-treated cells (0 μg/mL of biochanin A).

in MCF-7 cells was severely blocked to approximately 10% that in control cells by biochanin A at concentrations of 60 and 100 μg/mL even after only 1 day of treatment. We did not detect further inhibition when the cells were exposed to biochanin A for an additional 2 to 4 days. The concentration of 20 μg/mL inhibited DNA synthesis to a lesser extent. To further analyze the inhibitory effects of biochanin A at the early time points, MCF-7 cells were treated with biochanin A for 7 to 55 hours and the amount of de novo DNA

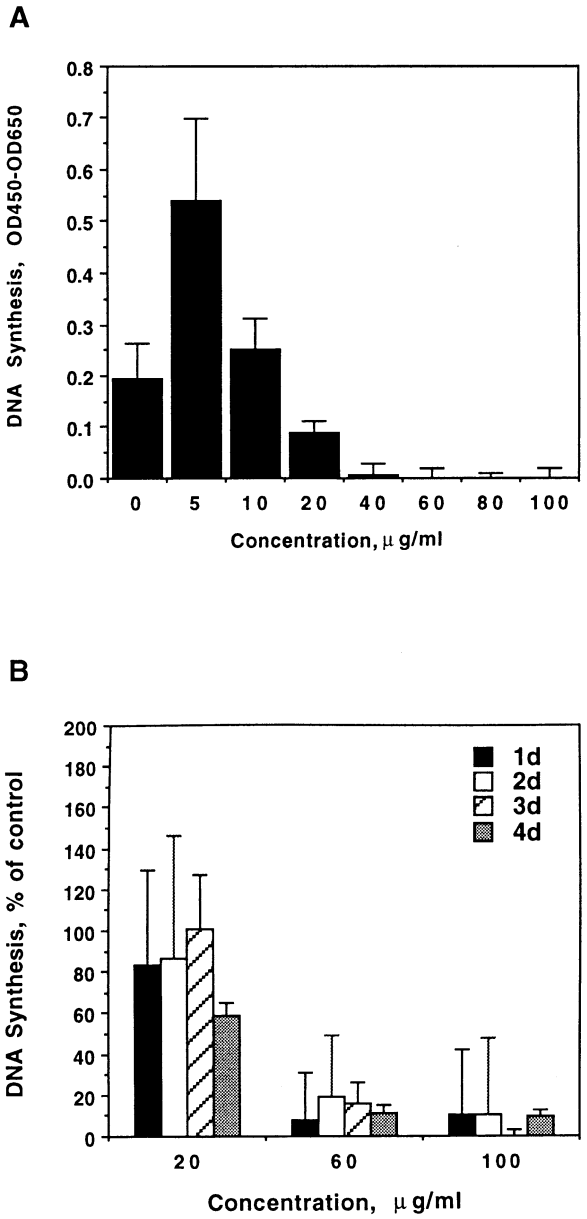

Figure 3 Regulation of DNA synthesis by biochanin A in MCF-7 cells. Cultured cells were treated with biochanin A at the concentrations indicated for *(A)* 4 days or *(B)* the period of time indicated, and the DNA synthesis was determined. Values are means ± SD.

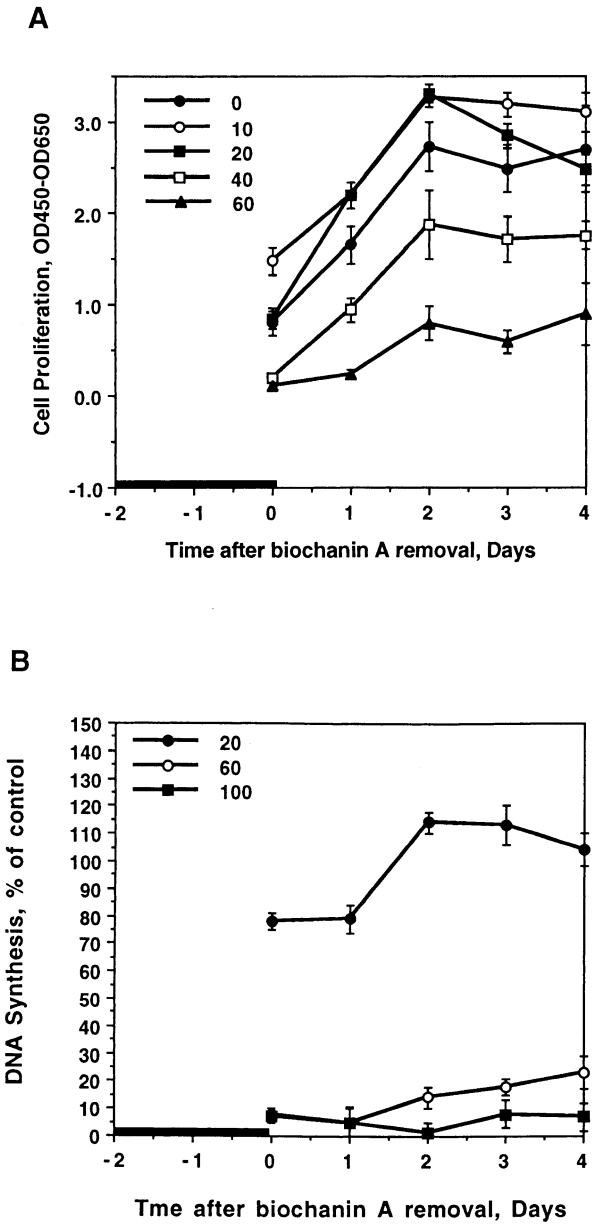

Figure 4 Reversibility in the inhibitory effect of biochanin A on cell proliferation and DNA synthesis in MCF-7 cells. Exponentially growing MCF-7 cells were treated with biochanin A at various concentrations for 48 hours. At the end of the incubation, biochanin A-containing medium was removed and cells were washed with fresh Dulbecco's modified Eagle's medium (DMEM) three times to remove any residual biochanin A. Fresh phenol red-free DMEM medium supplemented with 10% charcoal-stripped fetal bovine serum was added and *(A)* cell growth and *(B)* DNA synthesis were measured after cells were allowed to grow for 0 to 4 days. Values are means ± SD.

synthesis was determined (data not shown). At 60 μg/mL, significant inhibition in DNA synthesis was evident after cells had been treated with the compound for 7 hours.

Reversibility of the effects of biochanin A on MCF-7 cell proliferation and DNA synthesis

To ascertain the reversibility of the effect of biochanin A, cells were incubated with various concentrations of biochanin A for 48 hours and the compound was removed thereafter (*Figure 4*). At concentrations below 40 μg/mL, cells were able to showed regrowth after removal of the drug (*Figure 4A*). In contrast, at 60 μg/mL, MCF-7 cells failed to resume normal growth after the compound was removed for 2 days, although slight regrowth was occasionally observed after 3 days of drug removal. Analysis of the de novo DNA synthesis was done on cells treated with biochanin A (*Figure 4B*). At 60 and 100 μg/mL biochanin A concentrations, DNA synthesis did not return to normal compared with that of control cells for the period of time

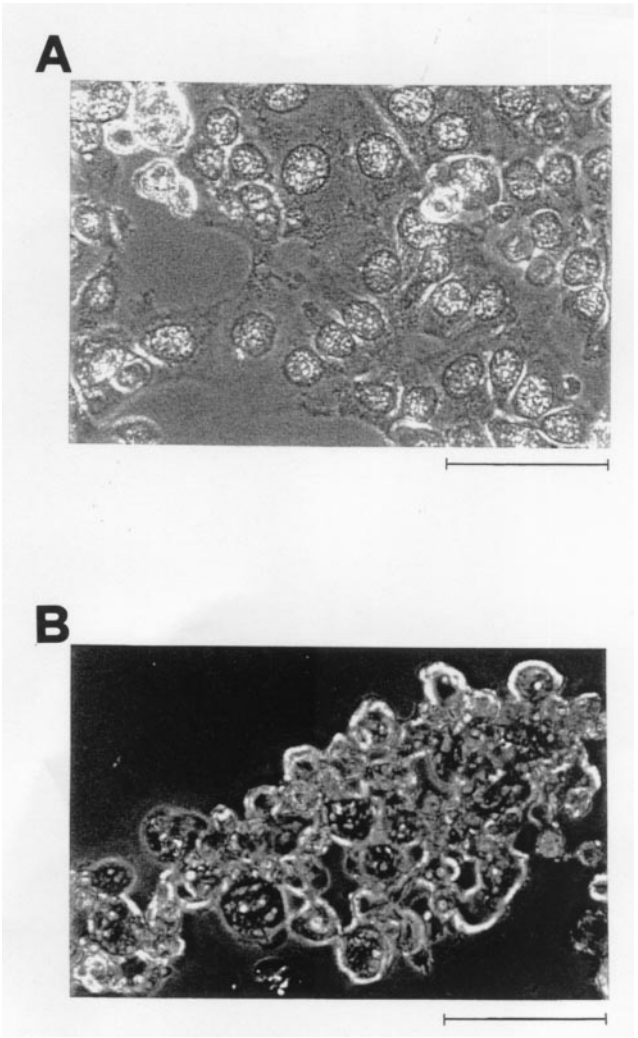

Figure 5 Cell morphology of biochanin A-treated cells. *(A)* Control MCF-7 cells that are attached to the surface of culture plates after being treated with dimethylsulfoxide in parallel and photographed for purposes of comparison. *(B)* After incubation with 100 μg/mL of biochanin A for 4 days, MCF-7 cells that were detached from the culture flask surface and became floated in the medium due to treatment were centrifugated, washed with phosphate buffered saline, reattached to the glass coverslip, and photographed. The bar below the photographs indicates a length for 50 μm.

examined. At 100 μg/mL, DNA synthesis was barely measurable, even when the compound had been removed for 4 days. Therefore, the inhibition of DNA synthesis at high doses of biochanin A was irreversible. At 20 μg/mL, DNA synthesis was reversible after biochanin A was removed.

Biochanin A treatment affects cell morphology

The morphology of MCF-7 cells treated with biochanin A was examined microscopically (*Figure 5*). Upon exposure to biochanin A for 4 days at 100 μg/mL, a significant portion of the cells became detached from the culture flasks and floated in the medium, although the percentage of floating cells was very low when the chemical was administered for only 1 day. After the cells floating in the medium

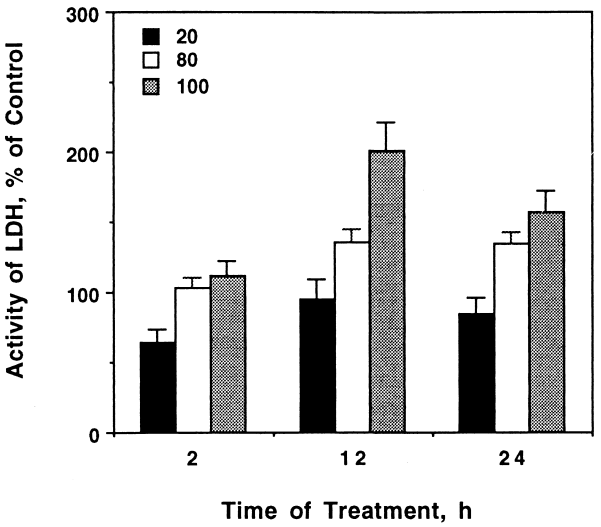

Figure 6 Cell membrane permeability of MCF-7 cells treated with biochanin A. MCF-7 cells were exposed to biochanin A at concentrations of 20, 80, or 100 μg/mL and incubated for 2, 12, or 24 hours. Lactate dehydrogenase (LDH) activity in the cell supernatant, which correlates with cell permeability, was determined as described in Materials and methods. The permeability of MCF-7 cells treated with dimethylsulfoxide for the same period of time was designated 100%. Values are means ± SD.

caused by the treatment were allowed to reattach to the cover slide surface, the cell morphology of the floating cells (*Figure 5B*) was compared with that of DMSO-treated control cells that remained attached to the culture plates (*Figure 5A*). A number of morphologic changes were clearly observed in the floating cells. All floating cells had became rounded, contained altered cellular material organization, and probably had lost integrity. They were not viable as determined by trypan blue dye exclusion assay. Although it appears that these cells underwent apoptosis, the loss of the nuclear membranes and the formation of apoptotic bodies, which are characteristics of apoptosis, were not easily identified due to the resolution of our assays (*Figure 5B*). Cells that remained attached to the flask surface after biochanin A treatment also exhibited altered cell morphology, although to a lesser extent compared with that of floating cells. They appeared to lose some cellular volume, but condensation of the cellular components were not as severe as that in cells that were found floating (data not shown). We did not observe similar morphologic changes in the DMSO-treated control cells (*Figure 5A*).

Biochanin A affects cell membrane permeability

To analyze the possible effects of biochanin A on cell morphology, we examined cell membrane permeability (*Figure 6*). This was measured by the amount of LDH released from the cells. The LDH is a cytoplasmic enzyme that is rapidly released into the cell culture supernatant upon changes in membrane permeability. Thus, an increase in the number of cells with damaged membrane results in increased LDH enzyme activity in the culture supernatant. After 12 hours of biochanin A treatment, the cell membrane began to show damage as indicated. At 100 μg/mL, bio-

chanin A was able to increase the cell membrane permeability to 157% that of control cells upon exposure to the chemical for 24 hours. The biochanin A-induced damage in the membrane of MCF-7 cells appears to be dose dependent. Changes in the cell membrane permeability reached a plateau at a 12 hours' incubation with biochanin A. At high concentrations of biochanin A (80 and 100 μg/mL), changes in the cell membrane permeability were more obvious than those at low concentrations (20 μg/mL).

Discussion

We have reported the cellular and molecular effects of the phytoestrogen biochanin A on estrogen-responsive MCF-7 cells. Results show that biochanin A differentially influences cell proliferation and DNA synthesis in MCF-7 cells. At low concentrations (<10 μg/mL), cell proliferation and DNA synthesis were stimulated by the treatment of biochanin A, whereas these events were inhibited by higher concentrations. The extent to which DNA synthesis was affected by biochanin A appears to be more profound than that of cell proliferation. This apparent discrepancy may be due to the fact that cells were not completely synchronized as expected. Higashi and Ogawara[20] reported that the phytoestrogen daidzein is involved in the regulation of G_1 phase initiation in the cell cycle. Flavopiridol, a flavone derivative, also was shown to induce a block at the G_1 phase in the cell cycle.[21] Biochanin A also may affect the cell cycle at the same point and influence only cells starting to enter the G_1-S phase in the cell cycle. In our assay, cells that were previously synchronized at the G_0-G_1 phase may have started to enter the cell cycle at different rates after a 24-hour preculture was performed to ensure attachment. Because DNA synthesis assays detect only newly-synthesized DNA that occurs in cells entering the S phase and cell proliferation assays measure viable cells in all phases of the cell cycle (i.e., G_1, S, G_2, and M), the effects of biochanin A would likely be more evident for DNA synthesis. Our observation also suggests that cell membrane damages in MCF-7 cells were produced by biochanin A. Cell membrane permeability was altered such that cell morphology had clearly changed. Perhaps the most striking features of these changes was the loss of cell shape and condensation of the cellular components. These morphologic changes suggest that apoptosis may be one of the means induced by biochanin A to cause cell death. Analyzed with flow cytometric techniques, Morris et al.[22] reported recently that genistein was capable of inducing apoptosis in human lymphoblastoid cells.

In our study, the concentrations of biochanin A (<10 μg/mL), which acted similarly to estrogen and stimulated the cell proliferation of target cells in vitro, is within the reported in vivo range (0.3–3 μg/mL) of circulating levels of phytoestrogen found in human consuming a soy-rich diet.[23,24] Our results confirm and expand on the results of recently reported investigations during the course of this study. The biphasic effects reported in this study are similar to those observed by Wang and Kurzer.[25] They showed the effects of phytoestrogens on DNA synthesis in MCF-7 cells to be variable and concentration dependent. Biochanin A enhanced the DNA synthesis at 0.1 to 10 μM and inhibited the synthesis with an IC_{50} value of 37.7 μM. Zava and Duwe[26] showed stimulation of DNA content to 150 to 400% at 0.001 to 10 μM of genistein and inhibition at 10 μM. At the concentration range of 0.6 to 0.1 μg/mL, Welshons et al.[27] found that the growth of MCF-7 cells as determined by DNA content was stimulated by phytoestrogen. Other reports have shown stimulation of protein content by daidzein at the concentration of 0.3 μg/mL.[28]

The IC_{50} values of 40 μg/mL observed in our studies is within the range reported previously. In MCF-7 cells, the IC_{50} for biochanin A for DNA synthesis was reported to be approximately 13 μg/mL.[25] The IC_{50} for genistein for cell growth was 10 μg/mL in MDA-468 cells.[29] Other phytoestrogens also showed similar range of IC_{50} values; the IC_{50} value for genistein in MCF-7 cells was 14 μg/mL and the IC_{50} value for daidzein has been reported to be 45 μg/mL.[25,29] Singhal et al.[30] showed an IC_{50} value of 18 μg/mL for quercetin in MDA-486 cells. Although it has been argued that the concentrations of phytoestrogen required to inhibit cell growth in vitro far exceed the highest plasma concentration of phytoestrogens that can be achieved in vivo (<3 μg/mL), little is know about the bioavailable concentrations of phytoestrogens in mammary epithelium tissues after consumption of a soy-rich diet.[31–33] Whether normal mammary epithelium tissues and ER positive cancer cells such as MCF-7 cells could potentially concentrate phytoestrogens through ER binding, cellular retention, or type II estrogen-binding sites remain currently unknown.[34] In addition, the plasma concentration of phytoestrogens was determined in humans. The concentration of phytoestrogens in the plasma of ruminants who consumed phytoestrogen-rich diet in a much larger amount and greater percentage than humans may be significantly higher than 3 μg/mL.

The full potency of the compound in the proliferation inhibition was achieved after exposure to the chemical for only 1 day. No noticeable further inhibition was evident. The inhibitory kinetics of biochanin A in cell proliferation were also present in DNA synthesis and cell membrane permeability. The irreversibility of biochanin A treatment at high concentrations may be correlated with infertility in animals that have been grazing estrogenic plants for long periods. This was the case in studies of sheep grazing estrogenic pastures.[35,36] Removal of these animals from the phytoestrogen-containing clover pastures did not reverse their progressively declining fertility. Our studies showed that cells did appear to recover from the influence of biochanin A at low concentrations. This may explain the results reported by Morley and associates,[5,37] who observed the recovery of temporary infertility in ewes grazing in highly estrogenic pastures after they had been transferred to a nonestrogenic diet. Temporarily infertility was found in ewes grazing highly estrogenic pastures at the time of mating and their fertility was recovered within a few weeks after the ewes were transferred to nonestrogenic pasture. Although no visible changes were induced in the ewes, a substantial reduction (up to 20%) in the number of ewes lambing often occurred, implying that the phytoestrogen-induced damage could occur without obvious morphologic changes.

The observation of the biphasic effects of biochanin A in

estrogen target cells is intriguing. In a natural setting, phytoestrogens function in plants as defensive compounds.[38] Perhaps they also serve as endogenous regulators of growth in determining pubertal onsets to optimize individual life histories in species-specific environments.[39] A previous report showed that concentrations of phytoestrogens increased in response to plant stressors such as mechanical injury, ultraviolet light, freezing, drought, or fungal infestation.[38] Animals that consume phytoestrogen-containing plants may exhibit suppressed reproduction due to the increased concentration of phytoestrogens. Thus, the presence of high concentrations of phytoestrogen could serve as clues to poor conditions for reproduction. Leopold et al.[40] observed that phytoestrogen concentrations in the food plants eaten by California quail increased during drought and subsequently suppressed reproduction. This response helps quails avoid reproducing in years in which vegetative growth is poor and offspring have a low probability of survival. The observed inhibition in cell proliferation of estrogen target cells that are exposed to high concentrations of biochanin A may have a correlation to such phenomena. In addition, the biphasic response in cell proliferation suggests that the biological actions of plant estrogens may be mediated through multiple pathways depending on the concentration of biochanin A. Previous studies have demonstrated the many mechanisms of phytoestrogen such as inhibition of tyrosine kinases, protein kinase C, and DNA topoisomerase II.[41–43] Recently, phytoestrogens also have been shown to induce apoptosis in culture cells and the reproductive tract of female rats.[22,44] The possible involvement of *c-myc*, a proto-oncogene product in these pathways, also may be worthy of consideration because the effects of an antiestrogen compound on estrogen-independent cells were mediated through *c-myc* overexpression.[45] Further investigations are necessary to clarify the mechanisms responsible for these effects.

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References

1 Beck, A. (1964). The oestrogenic isoflavones of subterranean clover. *Aust. J. Agr. Res.* **15,** 223–230
2 Martin, P., Horwitz, K., Ryan, D., and McCuire, W. (1978). Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology* **103,** 1860–1867
3 Miksicek, R. (1994). Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. *J. Steroid Biochem.* **49,** 153–160
4 Miksicek, R. (1995). Estrogenic flavonoids: Structural requirements for biological activity. *Proc. Soc. Exp. Biol. Med.* **208,** 44–50
5 Morley, F., Axelsen, A., and Bennett, D. (1966). Recovery of normal fertility after grazing on oestrogenic red clover. *Aust. Vet. J.* **42,** 204–206
6 McDonald, I. (1981). Detrimental substances in plant consumed by grazing ruminants. In *World Animal Sciences* (F. Morley, ed.), pp. 349–360, Elsevier Scientific Publishing Co., Amsterdam, The Netherlands
7 Perel, E. and Linder, H. (1970). Dissociation of uterotrophic action from implantation-inducing activity in two non-steroidal oestrogens (coumestrol and genistein). *J. Reprod. Fertil.* **21,** 171–175
8 Whitten, P., Russell, E., and Naftolin, F. (1994). Influence of phytoestrogen diets on estradiol action in the rat uterus. *Steroids* **59,** 443–449
9 Adlercreutz, H., Hockerstedt, K., Bannwart, C., Bloigu, S., Hamalainen, K., Fotsis, T., and Ollus, A. (1987). Effects of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG). *J. Steroid Biochem.* **27,** 1135–1144
10 Shutt, D. (1976). The effects of plant oestrogens on animal reproduction. *Endeavour* **35,** 110–113
11 Setchell, K., Gosselin, S., Welsh, M., Johnston, J., Balilistreri, W., Kramer, L., Dresser, B., and Tarr, M. (1987). Dietary estrogens–a possible cause of infertility and liver disease in captive cheetahs. *Gastroenterology* **93,** 225–233
12 Burroughs, C., Mills, K.T., and Bern, H.A. (1990). Long term genital tract changes in female mice treated neonatally with coumestrol. *Reprod. Toxicol.* **4,** 127–135
13 Leavitt, W. and Meismer, D. (1969). Sexual development altered by non-steroidal oestrogens. *Nature (London)* **218,** 181–182
14 Tang, B. and Adams, N. (1980). Effects of equol on oestrogen receptors and on synthesis of DNA and protein in the immature rat uterus. *J. Endocrinology* **85,** 291–297
15 Thompson, M., Lasley, B., Rideout, B., and Kasman, L. (1984). Characterization of the estrogenic properties of a nonsteroidal estrogen, equol, extracted from urine of pregnant macaques. *Biol. Reprod.* **31,** 705–713
16 Ying, C. and Gorski, J. (1994). DNA topology regulates rat prolactin gene expression. *Mol. Cell. Endocrinology* **99,** 183–192
17 Kaneko K., Gélinas, C., and Gorski, J. (1993). Activation of the silent progesterone receptor gene by ectopic expression of estrogen receptors in a rat fibroblast cell line. *Biochemistry* **32,** 8348–8359
18 SAS. (1990). *SAS/STAT® User's Guide* (Release 6.04), Vol. 2, SAS Institute Inc., Cary, NC, USA
19 Snedecor, G.W. and Cochran, W.G. (1980). *Statistical Methods*, 7th ed. Iowa State University Press, Ames, IA, USA
20 Higashi K. and Ogawara, H. (1994). Daidzein inhibits insulin- or insulin-like growth factor-1-mediated signaling in cell cycle progression of Swiss 3T3 cells. *Biochim. Biophys. Acta* **1221,** 29–35
21 Carlson, B., Dubay, M., Sausville, E., Brizuela, L., and Worland, P. (1996). Flavopiridol induced G_1 arrest with inhibition of cyclin-dependent kinase (CDK)2 and CDK4 in human breast carcinoma cells. *Cancer Res.* **56,** 2973–2978
22 Morris, S.M., Chen, J.J., Domon, O.E., McGarrity, L.J., Bishop, M.E., Manjanatha, M.G., and Casciano, D.A. (1998). p53, mutations, and apoptosis in genistein-exposed human lymphoblastoid cells. *Mutat. Res.* **31,** 41–56
23 Kirkman, L.M., Lampe, J.W., Campbell, D.R., Martini, M.C., and Slavin, J.L. (1995). Urinary lignan and isoflavonoid excretion in men and women consuming vegetable and soy diets. *Nutr. Cancer* **24,** 1–12
24 Xu, X., Wang, H-J., Murphy, P.A., Cook, L., and Hendrich, S. (1994). Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J. Nutr.* **124,** 825–832
25 Wang, C. and Kurzer, M.S. (1997). Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. *Nutr. Cancer* **28,** 236–247
26 Zava, D.T. and Duwe, G. (1997). Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. *Nutr. Cancer* **27,** 31–40
27 Welshons, W.V., Rottinghaus, G.E., Nonneman, D.J., Dolan-Timpe, M., and Ross, P.F. (1990). A sensitive bioassay for detection of dietary estrogens in animal feeds. *J. Vet. Diagn. Invest.* **2,** 268–273
28 Sathyamoorthy, N., Wang, T.T.Y., and Phang, J.M. (1994). Stimulation of pS2 expression by diet-derived compounds. *Cancer Res.* **79,** 957–961
29 Peterson, G. and Barnes, S. (1991). Genistein inhibition of the growth of human breast cancer cells: Independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Bioph. Res. Co.* **179,** 661–667
30 Singhal, R.L., Yeh, Y.A., Praja, N., Olah, E., Sledge, G.W. Jr., and Weber, G. (1995). Quercetin down regulates signal transduction in human breast carcinoma cells. *Biochem. Bioph. Res. Co.* **208,** 425–431
31 Goldin, B.R., Adlercreutz, H., Gorbach, S.L., Woods, M.N., Dwyer,

J.T., Conlon, T., Bohn, E., and Gershoff S.N. (1986). The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. *Am. J. Clin. Nutr.* **44,** 945–953

32 Adlercreutz, H., Markkanen, H., and Watanabe, S. (1993). Plasma concentrations of phyto-estrogens in Japanese men. *Lancet* **342,** 1209–1210

33 Morton, M.S., Wilcox, G., Wahlqvist, M.L., and Griffiths, K. (1994). Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. *J. Endocrinology* **142,** 251–259

34 Markaverish, B.M., Roberts, R.R., Alejandro, M.A., Johnson, G.A., and Middleditch, B.S. (1988). Bioflavonoid interaction with rat uterine type II binding sites and cell growth inhibition. *J. Steroid Biochem.* **30,** 71–78

35 Bennetts, H. (1946). Metaplasia in the sex organs of castrated male sheep maintained on early subterranean clover pastures. *Aust. Vet. J.* **22,** 70–78

36 Bennetts, H., Underwood, E., and Shier, F. (1946). A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Aust. Vet. J.* **22,** 2–12

37 Morley, F., Axelsen, A., and Bennett, D. (1964). Effects of grazing red clover (*Trifolium pratense L.*) during the joining season on ewe fertility. *Proc. Aust. Soc. Anim. Prod.* **5,** 58–61

38 Smith, D. and Banks, S. (1986). Biosynthesis, elicitation and biological activity of isoflavonoid phytoalexins. *Phytochemistry* **25,** 979–995

39 Bronson, F. and Rissman, E. (1986). The biology of puberty. *Biol. Rev.* **61,** 157–195

40 Leopold A., Erwin, M., and Oh, J. (1976). Phytoestrogens: Adverse effects on reproduction in California quail. *Science* **191,** 98–100

41 Kandaswami, C. and Middleton, E., Jr. (1994). Free radical scavenging and antioxidant activity of plant flavonoids. *Adv. Exp. Med. Biol.* **366,** 351–376

42 Alldrick, A.J., Flynn, J., and Rowland, I.R. (1986). Effects of plant-derived flavonoids and polyphenolic acids on the activity of mutagens from cooked food. *Mutat. Res.* **163,** 225–232

43 Geahlen, R.L., Koonvhanok, N.M., McLaughlin, L.I., and Pratt, D.E. (1989). Inhibition of protein tyrosine kinase activity by flavonoids and related compounds. *J. Nat. Prod.* **52,** 982–986

44 Tansey, G., Hughes, C.L., Cline, J.M., Krummer, A., Walmer, D.K., and Schmoltzer, S. (1998). Effects of dietary soybean estrogens on the reproductive tract in female rats. *Proc. Soc. Exp. Biol. Med.* **217,** 340–344

45 Kang, Y., Cortina, R., and Perry, R. (1996). Role of *c-myc* in tamoxifen induced apoptosis in estrogen-independent breast cancer cells. *J. Natl. Cancer I.* **88,** 279–284