The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A

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Abstract

The basis for the differential sensitivity of cultured normal human mammary epithelial (HME) cells and a transformed human breast cancer MCF-7 cell line to growth inhibition by the isoflavone genistein and its 4'-methyl ether derivative, biochanin A, was examined. In HME cells genistein is 5-fold more potent as a growth inhibitor than biochanin A, whereas in MCF-7 cells biochanin A and genistein are equally potent as growth inhibitors. Based on its properties as an in vitro protein tyrosine kinase (PTK) inhibitor, biochanin A would be expected to be a less potent growth inhibitor than genistein. To determine whether isoflavone metabolism could account for the observed differences in growth inhibition, metabolism experiments were conducted with HME and MCF-7 cells using [14C]genistein and [4-14C]biochanin A. MCF-7 cells extensively metabolized both isoflavones, producing two genistein metabolites with molecular weights of 350 and 380 and three biochanin A metabolites with molecular weights of 270, 350 and 380. In contrast, significant genistein or biochanin A metabolism was not observed in HME cells. Using mass spectrometry and nuclear magnetic resonance analysis, metabolite 350 from genistein and biochanin A experiments was identified as genistein 7-sulfate; biochanin A metabolite 270 was identified as genistein. Metabolite 380 was not unequivocally identified, but appeared to be a hydroxylated and methylated form of genistein sulfate. In MCF-7 cells, genistein 7-sulfate and metabolite 380 were detected primarily in the cell media fraction, suggesting that once formed these polar metabolites were excreted from the cells. These data show that isoflavone metabolism by transformed breast epithelial cells modulates the growth inhibitory effects of genistein and biochanin A. In MCF-7 cells, genistein metabolism was correlated with a decrease in growth inhibition, whereas biochanin A metabolism was associated with an increase in growth inhibition.

Introduction

In epidemiological studies, the consumption of soy isoflavones has been linked to decreased rates of several cancers, including breast and prostate cancer (1-4). Soy contains mg/g quantities of the isoflavones genistein (5,7,4'-trihydroxyisoflavone) and daidzein (7,4'-dihydroxyisoflavone), which are present as their β-glucoside conjugates (4). Several mechanisms for the anticancer effects of genistein have been proposed (5). These include topoisomerase II inhibition (6), induction of differentiation (7), inhibition of angiogenesis (8), and inhibition of protein tyrosine kinase (PTK*) activity (9).

PTK activity is critical for both normal and transformed cell growth (10). Therefore, compounds that inhibit PTK activity have been extensively investigated as potential anticancer agents (11,12). Furthermore, these compounds represent a novel class of drugs for pharmacologic intervention in the treatment and prevention of cancer that are directed against intracellular signaling components rather than DNA molecules (13). These data led our laboratory, and others, to hypothesize that consumption of soy isoflavones may be responsible for the decreased rates of cancer in soy consuming populations (14-16).

Although not present in soy foods, biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) is an isoflavone closely related to genistein, differing only in a methoxy group at the 4' position. In cell-free assays, biochanin A inhibits PTK activity 30-fold less effectively than genistein (9). However, in cell culture, biochanin A and genistein inhibit cancer cell growth approximately equally (17-19). In vivo, biochanin A decreased lung tumor incidence and multiplicity when administered to benzo[a]pyrene-treated, non-inbred Swiss-Webster mice (20). Furthermore, when administered in equal doses, biochanin A, but not genistein, inhibited the growth of several tumors derived from the human gastrointestinal tract and grown in BALB/c athymic nude mice (19).

In addition, we have previously reported that genistein and biochanin A are 10- and 6-fold more potent inhibitors, respectively, of EGF-stimulated growth of normal human mammary epithelial (HME) cells than of MCF-7 transformed human breast cancer cells (21,22,23). This increased sensitivity of non-transformed mammary epithelial cells to the inhibitory effects of genistein is consistent with its activity as a chemopreventive agent rather than a chemotherapeutic agent (5,22).

Although metabolism of isoflavones may largely occur in organs such as the liver, small intestine and kidney, isoflavone metabolism in the cells of the breast could explain the growth inhibitory action of biochanin A. It may also be responsible for the differential sensitivity of HME and MCF-7 cells to growth inhibition by genistein and biochanin A. Metabolism could influence isoflavone growth inhibition by altering the concentration or the chemical form of the isoflavones inside the cell.

In this study, the ability of HME and MCF-7 cells to metabolize [4-14C]genistein and [4-14C]biochanin A in cell culture was investigated. Radioactive metabolite peaks were isolated by HPLC; their identification was established by HPLC-mass spectrometry (HPLC-MS) and proton nuclear
magnetic resonance spectroscopy (1H-NMR). The consequences of metabolism of genistin and biochanin A are discussed as they relate to growth inhibitory effects of these isoflavones on transformed and non-transformed mammary epithelial cells.

Materials and methods

Materials

A genistin concentrate (40-50% genistin by weight) was a gift of Protein Technologies International (St Louis, MO). Fetal bovine serum, tissue culture media, supplements and antibiotics were obtained from Gibco (Gaithersburg, MD), Clonetics (San Diego, CA) or Upstate Biotechnology (Lake Placid, NY). Tissue culture supplies were from Costar (Charlotte, NC). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), biochanin A, and sulfatase (from Aerobacter aerogenes, no B-glucuronidase activity) were from Sigma Chemical Co. (St Louis, MO). Pre-stained molecular weight standards and SDS-PAGE chemicals were from BioRad (Richmond, CA). Immobilon PVDF membranes and Sep-Pak C18 cartridges were from Millipore (Bedford, MA). Sephadex G-25 and LH-20 were purchased from Pharmacia (Piscataway, NJ). Aquapore reversed-phase C4 columns were from Brownlee Labs (Santa Clara, CA). [4-14C]Genistein (23 mCi/mMol; 2.9 mg/ml) and [4-14C]Biochanin A (23 mCi/mMol; 1.2 mg/ml) were custom synthesized by Moravek Biochemicals Incorporated (Brea, CA) and had a radiochemical purity by HPLC of >98%, as assessed by reversed-phase HPLC. Goat anti-rabbit horse-radish peroxidase labeled antibody and Lumi-glo enhanced chemiluminescence reagents were from Kirkegaard and Perry Laboratories (Gaithersburg, MD). All other reagents were of highest grade available.

Cell culture

MCF-7 cells were a gift of Dr Craig Jordan (Northwestern University, Chicago, IL) and from the American Type Culture Collection (Rockville, MD). HME cells (strain 1001-7) were purchased from Clonetics Corporation (San Diego, CA). MCF-7 cells were maintained in Eagle's modified essential media (EMEM), HEPEES buffered, with 5% (v/v) fetal-bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). HME cells were maintained as described by the supplier.

Isoflavone isolation

Genistin was purified from genistin concentrate as described by Peterson and Barnes (17). In brief, genistin was purified by fractional crystallization and then subjected to acid hydrolysis to produce genistin. Genistin was recovered from unhydrolyzed genistin by ether extraction and recrystallized twice from 80% aqueous ethanol. It was >98% pure as judged by its UV absorbance at 262 nm and by reversed-phase HPLC.

Metabolism studies

Cells were plated in 6-well plates and grown to 70% confluence. [4-14C]Biochanin A or [4-14C]Genistein were added to the cells at a final concentration of 1 µM in 100% DMSO (0.5% v/v final DMSO concentration) (0.083 and 0.087 µCi/ml for biochanin A and genistin, respectively). Blank wells containing media and DMSO but no cells were used as controls. After incubation for the indicated times, media were collected and the cells were scraped and collected in 2 ml modified proteinase K buffer (25 mM EDTA, 137 mM NaCl and 10 mM Tris–HCl, pH 7.6). Cell lysates were sonicated and incubated with proteinase K (0.1 mg/ml) for 4 h at 37°C. Aliquots (10 µl) of cell lysate and lysate samples for analysis by reversed-phase HPLC were separated and purified as described by Falany et al. (24). Bacteria were pelleted and resuspended in bacterial lysis buffer (75 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 0.25 mM EDTA, 0.02 mg/ml lysozyme) for 20 min at 4°C. Bacteria were repelleted and resuspended in Triethanolamine (TEA) buffer (10 mM TEA, pH 7.5, 10% glycerol, 1.5 mM DTT, 10 µg/ml phenylmethylsulfonyl fluoride (PMSF) and sonicated (10 s, four times on ice). Bacterial cytosol was collected by centrifugation at 100,000 g for 45 min at 4°C. The cytosol was loaded onto a DEAEP-Sepharose Cl-6B (6 x 1 cm i.d.) column and the column was washed with 10 ml of 50 mM Tris–HCl, 0.1 M NaCl, 0.1% Tween 20 to remove DMSO. The column was next washed with 10 ml TEA buffer containing 100 mM NaCl and then P-PST was eluted with a gradient of 0-25% TEA buffer over 5 min. The fractions containing P-PST activity were pooled and stored at -70°C.

Enzymatic synthesis of genistein sulfate

Sulfation of genistein was performed using a partially purified baculovirus-expressed phenol sulfating form of human phenol sulfotransferase (P-PST) enzyme. Phenol sulfoanalogues of genistein and biochanin A were synthesized using the E. coli expression system. Enzymatic genistein sulfate was prepared by incubating 850 µg genistein in sulphate buffer (50 mM Tris–HCl, pH 7.4, 10 mM MgCl2, 50 µM 3′p-hydroxybenzoic-4′-sulfophosphate (PAPS), and P-PST). The reaction mixture was analyzed by HPLC and mass spectrometry to determine the formation of genistein sulfate.

Nuclear magnetic resonance spectroscopy

Genistin and the enzymatic and synthetic genistein sulfates (20-30 µg) were dissolved in DMSO-d6 (0.5 ml). 1H and 13C NMR spectra were obtained in overnight data collections (14 h) with a 6173-Hz sweep width, 2.65-s repetition rate, and 30° sweep angle on a Bruker ARX spectrometer (operating frequency of 300 MHz). Spectra were internally referenced to the DMSO proton resonance (2.49 ppm).

Western blot analysis

HME and MCF-7 cells were grown as described to 90% confluence. Media were aspirated and cells were lysed immediately in SDS-sample buffer.
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(0.1% SDS, 1% triton X-100, 137 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM EDTA and 1 mM sodium orthovanadate). The plates were rocked at room temperature for 30 s and cells were scraped into microfuge tubes. The supernatant fraction was passed through a 21 gauge needle eight times to shear DNA and centrifuged for 10 min at 10 000 g. Protein concentrations were determined by the Lowry method (25). Cell lysate proteins (800 μg) were resolved on 8% SDS–PAGE and transferred to nitrocellulose membranes. Immunoblot analysis of P-PST was performed using a rabbit anti-human P-PST antibody (1:10 000) (26) and visualized using enhanced chemiluminescence as described by the manufacturer (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Results

MCF-7 cells metabolize genistein and biochanin A

A metabolite peak (2G) was detected by scintillation counting of reversed-phase HPLC eluate fractions when [4-14C]genistein was incubated with MCF-7 cells over a 4-day period (Figure 1A). This peak was not present when genistein was incubated in the presence of cell culture media alone without MCF-7 cells (Figure 1B). An additional peak was observed by reversed-phase HPLC using UV detection, but it was not radioactive, suggesting that the compound(s) in this peak originated from a cell lystate component, rather than genistein. Additionally, this peak was present when cells were in grown media alone in the presence or absence of DMSO (data not shown).

MCF-7 cells extensively metabolized biochanin A over a 4-day incubation, producing two new peaks (2B and 3B) (Figure 2A). Again, none of these peaks were present when biochanin A was incubated in cell culture media in the absence of MCF-7 cells (Figure 2B). As for the experiments with genistein, an additional peak was observed by reversed-phase HPLC using UV detection and appeared to be derived from a cell media component (non-radioactive) (data not shown). Co-chromatography experiments demonstrated that peaks 2B and 3B had the same HPLC mobility as peaks 2G, and genistein, respectively (data not shown). These data suggested that MCF-7 cells initially demethylated biochanin A, converting it to genistein. Once formed, genistein was then further metabolized by MCF-7 cells as described above. Similar results were obtained for genistein and biochanin A metabolism with four other transformed mammary epithelial cell lines (G.Peterson, G.-P.Ji, M.Kirk and S.Barnes, unpublished data).

The metabolites of genistein and biochanin A, peaks 2B and 2G, were found primarily in cell culture media fractions of MCF-7 cells, with trace amounts being recovered in cell lystate fractions (Figures 1C and 2C). The amount of peaks 2B and 2G detected in the cell lystate fraction was at the limit of HPLC-UV sensitivity, but could be detected by scintillation counting of HPLC eluates. These data suggested that as soon as peaks 2B and 2G were formed, they were efficiently excreted from MCF-7 cells. Genistein and biochanin A were also detected in cell lystate fractions at levels roughly equivalent to that of peaks 2B and 2G (Figures 1C and 2C). In experiments with biochanin A, genistein was the primary isoflavone detected in the cell lysters (Figure 2C).

Identification of metabolites

Identification of the metabolites of genistein and biochanin A was carried out using a combination of reversed-phase HPLC-MS and NMR. Peak 2G when analyzed by reversed-phase HPLC-electrospray ionization-MS was composed of two distinct compounds, with mass to charge ratio (m/z) values for the [M-H]− ions of 349 (metabolite 350) and 379 (metabolite 380) (Figure 3A and B), with metabolite 350 being the major compound produced. These compounds migrated with almost exactly the same retention time on HPLC, indicating that the compounds were similar in structure. Similar data were obtained with peak 2B (data not shown). Both compounds in peak 2G originated from genistein, as the ratio of the intensities of the [M-H]− molecular ion and the [M-H+2]− isotope ion for the separate metabolites were similar to that of the [14C]genistein starting material (Figure 3 insert). A similar isotopic ratio was obtained for metabolite 350 in peak 2B (data not shown).

For metabolite 350, the increase in molecular weight from...
genistein (+80) corresponds to the addition of a single sulfate group. This was verified by the sensitivity of metabolite 350 to the hydrolytic action of sulfatase, but not glucuronidase (data not shown). Additionally, the product of the sulfatase reaction had the same HPLC retention time as authentic genistein (Figure 4A and B).

To further confirm that metabolite 350 was a genistein monosulfate, genistein sulfate was both chemically synthesized and prepared from an in vitro reaction using bacterially expressed and purified phenol sulfating form of human phenol sulfotransferase (P-PST). The principal synthetic genistein monosulfate had an HPLC retention time similar to, but different from, metabolite 350 (Figure 5A), whereas the minor synthetic genistein monosulfate had the same retention time as metabolite 350 (Figure 5A). On the other hand, the genistein sulfate produced enzymatically by the P-PST, migrated with the same retention time as metabolite 350 (Figure 5B). These data suggested that metabolite 350 and the P-PST-produced genistein sulfate were identical, while the principal synthetic genistein monosulfate was another genistein sulfate isomer.

To address the site of sulfate attachment in metabolite 350, the principal synthetic genistein sulfate, P-PST-produced genistein sulfate, and genistein were analyzed by $^1$H-NMR. When the proton chemical shifts for the enzymatically produced genistein sulfate were compared with those for genistein, the $H_6$ and $H_8$ proton resonances underwent chemical shift changes of 0.5 p.p.m. and the 7-OH resonance was absent, whereas the $H_3'$ and $H_5'$ proton resonances were unchanged (Table I). These alterations to the $^1$H-NMR spectrum indicated that the enzymatically produced genistein monosulfate was the 7-isomer. On the other hand, when the proton chemical shifts for the principal synthetic genistein monosulfate and genistein were compared, the $H_3'$ and $H_5'$ proton resonances underwent chemical shift changes of 0.4 p.p.m. (Table I), whereas the $H_6$ and $H_8$ proton resonances were unchanged, indicating that the sulfate is located at the 4'-position in synthetic genistein sulfate.

Peak 3B from biochanin A metabolism experiments migrated with a retention time identical to that of genistein, suggesting that MCF-7 cells demethylated biochanin A and produced genistein. This was confirmed as peak 3B and genistein co-migrated on HPLC (Figure 6) and gave identical spectra in HPLC-MS experiments (data not shown).

**HME cells do not metabolize genistein and biochanin A**

In contrast to MCF-7 cells, HME cells did not significantly metabolize genistein (Figure 7A and B) or biochanin A (Figure 8A and B) over a 3-day incubation period, as judged by scintillation counting of HPLC eluates. Additionally, HPLC-UV detection did not detect metabolite formation, and HPLC-MS analysis of medium extracts confirmed HME cells did not produce significant quantities of the metabolites observed in media from experiments with MCF-7 cells (data not shown). In some experiments, minor peaks and traces of radioactivity were detected eluting at 8 to 9 min in HME cells, but the amount detected was always at least 20-fold lower than that observed in MCF-7 cells (data not shown). Similar results were obtained with batches of HME cell lines from different donors (Peterson and Barnes, unpublished data).

Significant quantities of genistein or biochanin A metabolites were not detected in HME cell lysates (Figures 7C and 8C). However, there was consistently more genistein and biochanin A in the cell lysate fraction from HME cells than from MCF-7 cells as judged by the amount of radioactivity recovered (Figure 1C versus 7C and 2C versus 8C).

**Expression of P-PST correlates with genistein metabolism**

Sulfotransferases are known to be expressed in breast tissue and to sulfate small planar molecules such as estrogens and estrogen-like compounds (i.e. genistein). A specific human sulfotransferase, P-PST, is expressed in MCF-7 cells (27). Since metabolite 350 appeared to be identical to P-PST-produced genistein sulfate, we investigated the expression of P-PST in MCF-7 and HME cells. Whole cell lysates of MCF-7 and HME cells were analyzed by Western blot analysis using a rabbit polyclonal anti-PST antibody (26). A single band

![Fig. 2. Reversed-phase HPLC of $^{14}$C-radioactivity in MCF-7 cell culture media and lysate fractions. (A) Media from incubation of biochanin A with MCF-7 cells; (B) media from incubation of biochanin A in the absence of MCF-7 cells; and (C) cell lysate from incubation of biochanin A with MCF-7 cells.](https://academic.oup.com/carcin/article-abstract/17/9/1861/337970)
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Fig. 3. HPLC analysis of peak 2G. Peak 2G was analyzed by reversed-phase HPLC-electrospray ionization (ESI)-MS and selected ion chromatograms (A) m/z 349 and (B) m/z 379, were prepared from the collected data. (Insert) Mass spectra of the peaks identified in selected ion chromatograms show that both metabolites in peak 2G originate from genistein. Note the [M-H]/[M-H + 2] ratios for m/z 349 and 379 from peak 2G (A) and m/z 269 from [14C]genistein (B) are similar.

Fig. 4. MS-MS spectra of (A) the hydrolyzed product from the treatment of metabolite 350 with sulfatase; and (B) authentic genistein.

corresponding in molecular weight to recombinant P-PST, was detected in MCF-7 cells, but not in HME cells, and this band co-migrated with bacterially expressed P-PST (Figure 9).

Discussion

These studies have shown that genistein and biochanin A are metabolized by MCF-7 cells, but not by HME cells. From the data presented, a general scheme of metabolism in MCF-7 cells has been deduced. Genistein is metabolized by MCF-7 cells, yielding two monosulfated metabolites derived from genistein, metabolite 350 and metabolite 380. Metabolism of biochanin A proceeds by a similar mechanism. As a first step in its metabolism, biochanin A is metabolized to genistein via a demethylation reaction and the generated genistein is metabolized further to metabolite 350. The sulfated metabolites are excreted from the cell, ultimately terminating the growth inhibitory effects of genistein. Therefore, metabolism may explain the effects of genistein and biochanin A on inhibition of MCF-7 and HME cell growth.

The idea that isoflavones and PTK inhibitors themselves,
media (data not shown). However, HPLC-MS and NMR which could serve as sites of sulfation to produce metabolite Genistein has hydroxyl groups at the 5, 7 and 4' positions, previously reported.

...culture (29). Substantial metabolism of genistein and biochanin...in vitro reported loss of specificity when analyzed in cell...

...Cl OH resonance was absent, indicating sulfation occurred at the C7 OH. Genistein sulfate, synthesized genistein sulfate. Note the proton resonances at the H3' and H5' positions underwent chemical shifts for the chemical genistein sulfate, indicating sulfation occurred at the C4' OH. For P-PST genistein sulfate, the H6 and H8 proton resonances underwent chemical shift changes and the C7 OH resonance was absent, indicating sulfation occurred at the C7 OH. The structure of metabolite 380 is more complex, since in addition to sulfation, seven positions on the genistein molecule are potentially open for hydroxylation and two sites for methylation. Attempts to isolate enough metabolite 380 for NMR analysis have been unsuccessful.

Metabolism of genistein and biochanin A is important in understanding the inhibitory effects of these isoflavones on MCF-7 and HME cell growth. Genistein and biochanin A inhibit the growth of MCF-7 cells stimulated by fetal bovine serum with IC50 values of 9.7 and 7.0 μg/ml, respectively (23). In contrast, HME cells stimulated by bovine pituitary extract are more sensitive to growth inhibition by genistein and biochanin A with IC50 values of 0.9 and 3.3 μg/ml, respectively (Peterson and Barnes, unpublished). These data show that genistein and biochanin A are more potent inhibitors of HME cell growth than MCF-7 cell growth.

MCF-7 cell lysates contained low and variable amounts of genistein 7-sulfate (met 350), metabolite 380, genistein, and biochanin A as determined by scintillation counting of HPLC eluates. The majority of radioactivity, in the form of genistein 7-sulfate and metabolite 380, was found almost exclusively in MCF-7 cell media. Since 60-100% of biochanin A is converted to genistein, and 25-75% of genistein is converted to genistein 7-sulfate, the result of the excretion of genistein 7-sulfate is to maintain a lower concentration of genistein in MCF-7 cells.

In contrast, since no significant metabolism occurs in HME cells, the intracellular concentrations of genistein and biochanin undergo metabolism in target cells is not new. Evidence is accumulating that tyrphostins, synthetic PTK inhibitors, undergo metabolism in cells (28,29). This could explain the long incubation times necessary for tyrphostin action and the reported loss of in vitro specificity when analyzed in cell culture (29). Substantial metabolism of genistein and biochanin A has also been observed in animals (30) and humans, although metabolism by specific cell types have not been previously reported.

The predominant metabolite of genistein and biochanin A in MCF-7 cells is genistein 7-monosulfate, metabolite 350. Genistein has hydroxyl groups at the 5, 7 and 4' positions, which could serve as sites of sulfation to produce metabolite 350. NMR experiments conducted on metabolite 350 isolated from cell media to determine the position of sulfation did not settle this issue due to unresolvable contaminants in the cell media (data not shown). However, HPLC-MS and NMR studies with P-PST-derived and chemically synthesized genistein monosulfate strongly suggested that metabolite 350 was sulfated at the 7-position. This conclusion is supported by the apparent hydrophobicity of these genistein sulfates as compared to daidzein. The 5-hydroxyl group of genistein (and genistein 4'- and 7-sulfates) is located near a ketone oxygen at the 4'-position. These two groups form a hydrogen bond, giving genistein a more hydrophobic nature than expected, as demonstrated by its longer elution time than daidzein (which lacks the 5-hydroxyl group) on reversed-phase HPLC (31,32). Metabolite 350, even with the addition of a charged sulfate group, still elutes from reversed-phase HPLC more slowly than daidzein, indicating that the hydrophobic nature of the molecule is not altered. This alone suggests that sulfation does not occur at the 5'-position.

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Fig. 7. Reversed-phase HPLC of $^{14}$C-radioactivity in HME cell culture media and lysate fractions. (A) Media from incubation of genistein with HME cells; (B) media from incubation of genistein in the absence of HME cells; and (C) cell lysate from incubation of genistein with HME cells.

Fig. 8. Reversed-phase HPLC of $^{14}$C-radioactivity in HME cell culture media and lysate fractions. (A) Media from incubation of biochanin A with HME cells; (B) media from incubation of biochanin A in the absence of HME cells; and (C) cell lysate from incubation of biochanin A with HME cells.

Fig. 9. P-PST expression correlates with genistein and biochanin A metabolism. Whole cell lysates (800 μg protein) of (A) MCF-7 cells, (B) HME cells and (C) 1.3 μg bacterially expressed P-PST were probed with anti-P-PST polyclonal antibody and visualized by enhanced chemiluminescence (see Materials and methods, Western blot analysis).

A should be unaffected. HME cell lysates always contained more genistein and biochanin A than MCF-7 cell lysates, suggesting the intracellular isoflavone concentrations were higher in HME cells. The amount of genistein, biochanin A and genistein 7-sulfate detected in cell lysates could, in part, be the result of the non-specific binding of these compounds to the exterior of the cells and is not a true indicator of intracellular concentration. However, preliminary experiments showed HME cells do have higher intracellular concentrations than MCF-7 cells (Peterson, Kirk and Barnes, unpublished data).

These data suggest that the differential sensitivity of HME
and MCF-7 cells to genistein and biochanin A growth inhibition could be due to the intracellular formation of genistein 7-sulfate and metabolite 380 and their excretion from MCF-7 cells. This hypothesis is consistent with the role of sulfation in terminating the biological effects of compounds (33). In this mechanism, sulfation serves to signal the efflux of genistein from the cell.

The metabolism of biochanin A to form genistein is also significant when the effect of biochanin A on the growth of HME and MCF-7 cells is examined. Biochanin A is a 30-fold less potent PTK inhibitor than genistein (9). Biochanin A, however, is a more potent growth inhibitor of MCF-7 and human prostate cancer cells (17,18,22,23). Since at least 60–100% of the biochanin A added to MCF-7 cells is metabolized to genistein, adding biochanin A to MCF-7 cells is equivalent to adding genistein. Although the genistein is ultimately converted to genistein-7-sulfate and excreted, time course experiments show conversion of biochanin A to genistein occurs within 24 h, while conversion of genistein to genistein-7-sulfate is slower (genistein is present after 36 h). This suggests biochanin A-derived genistein has a significant residence time inside MCF-7 cells in which it can inhibit cell growth. The greater hydrophobicity of biochanin A compared with genistein (due to its methylation at the 4'-OH) may also augment its uptake into the cell and/or cellular distribution, facilitating genistein growth inhibition. The increased cytotoxicity of genistein in MCF-7 cells supports this hypothesis (23). Therefore, biochanin A metabolism to genistein provides an explanation for the increased effects of biochanin A on cell growth than would be expected from its in vitro PTK inhibitory activity. Since HME cells do not convert biochanin A to genistein, biochanin A is a weaker growth inhibitor than genistein. Similar metabolic conversions may be responsible for the action of biochanin A in other cell culture and animal models (19,20).

Breast epithelial cells express several types of sulfotransferases. Of the sulfotransferases in MCF-7 cells, only P-PST is capable of sulfating genistein in vitro (27). The levels of P-PST in MCF-7 and HME cells (as determined by Western blot analysis) correlated with metabolism of genistein to genistein sulfate. These data strongly suggest that P-PST, or a very similar enzyme, is responsible for conversion of genistein to genistein sulfate in MCF-7 cells. In MCF-7 cells, genistein is converted to two sulfated isoflavone metabolites. These data strongly suggest that P-PST, or a very similar enzyme, is capable of sulfating biochanin A. In MCF-7 and HME cells, biochanin A is converted to genistein sulfate. These data strongly suggest that P-PST, or a very similar enzyme, is responsible for conversion of genistein to genistein sulfate in MCF-7 cells.

In vitro

The role of genistein as a growth inhibitor is complicated by the metabolism of biochanin A to genistein. Biochanin A is converted to genistein 7-sulfate, providing genistein sufficient time to inhibit MCF-7 cell growth. HME cells do not metabolize genistein or biochanin A. Therefore, higher intracellular isoflavone concentrations are reached in HME cells than in MCF-7 cells at equivalent extracellular concentrations, allowing growth inhibition to occur at lower isoflavone concentrations. Also, since biochanin A is not metabolized in HME cells, its effect on HME cell growth is less than that of genistein.

Acknowledgements

A genistein concentrate, used in the isolation and purification of genistin and genistein, was kindly donated by Protein Technologies International, a St Louis, MO-based Company. We gratefully acknowledge the help of Dr Don Muccio, Department of Chemistry, UAB, who carried out the NMR experiments, and Michelle Smith and Guoping Ji for their excellent technical assistance. These studies were supported in part by grants from the American Institute for Cancer Research (91B58), the National Cancer Institute (SRO1 CA-61668), the Nebraska Soybean Promotion and Utilization Board, and the United Soybean Board. The mass spectrometer was purchased by funds from a NIH Instrumentation Grant (S10RR06487) and from this institution. Operation of the UAB Comprehensive Cancer Center Mass Spectrometry Shared Facility has been supported in part by a NCI Core Research Support Grant to the UAB Comprehensive Cancer (P30 CA13148).

The data herein were presented in part at the 1994 Annual Meeting of the American Society for Cell Biology, San Francisco, and have appeared in abstract form (Mol. Biol. Cell, 5 [Suppl.], abstract 2232). The data were also published as a requirement for the partial fulfilment of a PhD dissertation from the University of Alabama at Birmingham for TOP.

References


Received on April 4, 1996; revised on June 7, 1996; accepted on June 11, 1996.