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Abstract

White button mushrooms (*Agaricus bisporous*) are a potential breast cancer chemopreventive agent, as they suppress aromatase activity and estrogen biosynthesis. Therefore, we evaluated the activity of mushroom extracts in the estrogen receptor–positive/aromatase-positive MCF-7aro cell line *in vitro* and *in vivo*. Mushroom extract decreased testosterone-induced cell proliferation in MCF-7aro cells but had no effect on MCF-10A, a nontumorigenic cell line. Most potent mushroom chemicals are soluble in ethyl acetate. The major active compounds found in the ethyl acetate fraction are unsaturated fatty acids such as linoleic acid, linolenic acid, and conjugated linoleic acid. The interaction of linoleic acid and conjugated linoleic acid with aromatase mutants expressed in Chinese hamster ovary cells showed that these fatty acids inhibit aromatase with similar potency and that mutations at the active site regions affect its interaction with these two fatty acids. Whereas these results suggest that these two compounds bind to the active site of aromatase, the inhibition kinetic analysis indicates that they are noncompetitive inhibitors with respect to androstenedione. Because only conjugated linoleic acid was found to inhibit the testosterone-dependent proliferation of MCF-7aro cells, the physiologically relevant aromatase inhibitors in mushrooms are most likely conjugated linoleic acid and its derivatives. The *in vivo* action of mushroom chemicals was shown using nude mice injected with MCF-7aro cells. The studies showed that mushroom extract decreased both tumor cell proliferation and tumor weight with no effect on rate of apoptosis. Therefore, our studies illustrate the anticancer activity *in vitro* and *in vivo* of mushroom extract and its major fatty acid constituents. (Cancer Res 2006; 66(24): 12026-34)

Introduction

The female hormone estrogen plays a critical role in breast cancer development by binding to the estrogen receptor and inducing the expression of peptide growth factors that are responsible for the proliferation of cancer cells. Approximately 60% of premenopausal and 75% of postmenopausal patients have estrogen-dependent carcinomas. Therefore, abnormal expression of aromatase, the enzyme responsible for the synthesis of estrogen, in breast cancer cells and/or surrounding adipose stromal cells may have a significant influence on tumor development and growth in breast cancer patients. Recent clinical trials have shown aromatase inhibitors to be effective in the treatment of hormone-responsive breast cancer and to significantly prevent contralateral cancers (1–3). These results indicate that suppression of estrogen formation in postmenopausal women by aromatase inhibitors may be a useful prevention/treatment strategy in these women.

Whereas pharmaceutical agents have a therapeutic role in cancer, dietary constituents should be explored for their preventive potential. Therefore, our laboratory initiated a research project to investigate anti-aromatase phytochemicals in common vegetables that may lead to the suppression of breast cancer cell proliferation. Our experiments revealed that of the seven vegetable extracts tested, the extract of white button mushroom was the most effective in inhibiting the activity of human placental aromatase (4). Of the other extracts evaluated, celery had a modest inhibitory effect. Extracts prepared from green onion, carrot, bell pepper, broccoli, and spinach did not exhibit significant aromatase inhibition under these experimental conditions. To determine whether aromatase inhibition was unique to the white button mushroom, 10 additional varieties of mushrooms were evaluated (4). The results identified the stuffing mushroom as the most potent inhibitor of aromatase activity; however, the shiitake, white button mushroom, portobello, crimini, and baby button mushrooms also showed significant inhibitory effects. These findings suggest that a number of varieties of mushroom possess inhibitory effects on aromatase activity. Because it is the most common type of mushroom consumed by the general population, we studied the anticancer potential of heat-stable extracts from white button mushroom. Our results led us to hypothesize that the white button mushroom contains potential chemopreventive agents active against hormone-dependent breast cancer.

In this study, experiments were done to identify and characterize the anti-aromatase chemicals in white button mushroom. We also did cell culture experiments to confirm the inhibitory effect of mushrooms against the proliferation of breast cancer cells. In addition, animal experiments were carried out to determine the anti-aromatase action of mushrooms *in vivo*. Prevention strategies involving mushrooms are certainly readily available, affordable, and acceptable to women. The information gained from our studies is important as it could aid in the design of more highly developed and effective breast cancer prevention strategies involving mushrooms.

Materials and Methods

Materials. Progesterone, NADPH, standard fatty acids [myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, conjugated linoleic acid (a mixture of *cis*- and *trans*-9,11- and -10,12 isomers), and linolenic acid], and fatty acid methyl ester (oleic acid methyl ester, linoleic acid methyl

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ester, and linolenic acid methyl ester) were purchased from Sigma Chemical Co. (St. Louis, MO). Fatty acid standards including conjugated linoleic acid isomers were obtained from Matreya, Inc. (Pleasant Gap, PA). [1-β-^{3}H]Androstenedione was purchased from NEN-Dupont (Boston, MA). Organic solvents for extraction and column chromatography were of high-performance liquid chromatography grade and were purchased from Burdick & Jackson (Muskegon, MI). For gas chromatography-mass spectroscopy (GC-MS) analyses, bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane reagent was purchased from Supelco (Bellefonte, PA). Fresh white button mushrooms (*Agaricus bisporus*) were purchased from local supermarkets and some were donated by the Mushroom Council (New York, NY). Microsomes were prepared from human placenta as previously described (5).

Cell culture. Chinese hamster ovary (CHO) cells were cultured in Ham's medium, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 1×-penicillin/streptomycin, 15 mmol/L HEPES, and 10% fetal bovine serum (FBS) optimized for CHO cells (Hyclone, Logan, UT). MCF-7aro cells were cultured in Eagle's MEM with Earle's salts, 1 mmol/L pyruvate, 2 mmol/L glutamine (Irvine Scientific, Santa Ana, CA), and 10% heat-inactivated FCS (ΔFCS; Omega Scientific, Tarzana, CA). MCF-10A cells were cultured in 50:50 DMEM/HAM's F-12 supplemented with 10% horse serum, 2 μmol/L insulin, 0.5 mg/L hydrocortisone, 20 μg/L epidermal growth factor, and 100 μg/L chloeratoxin. All cell lines were incubated in a humidified environment at 37°C in 5% CO_2.

Preparation of mushroom extract. The mushroom extraction began with 200 pounds of fresh white button mushrooms that were chopped, boiled, and filtered to give a broth. The filtered broth was concentrated to 10 liters *in vacuo* at 45°C, and the solid material was extracted with methanol (2 × 10 liters), concentrated *in vacuo* at 45°C. Both concentrated extracts were combined and partitioned with ethyl acetate (3 × 10 liters) and generated an ethyl acetate fraction (EA; 86.4 g) and aqueous residue (Aq; 5.50 kg). To produce the 6× mushroom extract, 60 g of white button mushrooms were chopped and boiled; 15 mL of 4 kg/L concentrated mushroom water extraction were applied to 5 g/60 mL capacity polyamide column (Discovery DPA-6S SPE, Supelco). Fractions were eluted by a step gradient (50 mL of each step) of increasing methanol to water. The 20% methanol-water fraction (similar to EA2) was rotor evaporated to dryness and then redissolved in 1 mL of water to produce the 6× mushroom extract. Therefore, 6 g of mushrooms can produce 100 μL of 6× fraction.

Extraction and identification of anti-aromatase chemicals. The EA fraction was subjected to column chromatography on polyamide resin (Discovery DPA-6S, Supelco) using H_2O-methanol mixture (0–100% methanol, step gradient) as mobile phase to afford four fractions (EA1–EA4; eluted at 0%, 30%, 50%, and 100% methanol, respectively). Of these, EA2 (714 mg) showed the most potent aromatase inhibition. Additional chromatographic separation of EA2 (600 mg) on silica gel (220–440 mesh, Fluka, Buchs, Switzerland), using *n*-hexane/acetone mixture (4:1, v/v)-acetone 100%, gave 13 fractions (EA2-1–EA2-13). Among them, fraction EA2-2 (eluted between 26% and 32% acetone; 15.1 mg) showed the most potent activities, and the constituents were analyzed using GC-MS after being converted to tetramethylsilyl derivatives. To evaluate the activity of aqueous fraction, 1.0 g of Aq was boiled in 80% ethanol and stored at 4°C for 1 day to afford polysaccharide precipitates (148 mg) and soluble residue (844 mg).

GC-MS analyses. The aromatase-inhibiting constituents from the nonpolar fractions of the mushroom extract and standard fatty acids were analyzed using GC-MS method. One milligram of each sample was dissolved in 100 μL of pyridine/bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane reagent (1:1, v/v), incubated at 50°C for 30 minutes to convert into silylated forms, and analyzed on a fused silica capillary column (ultra-2, 25 m × 0.25 mm i.d., 0.33 μm, Agilent Technologies, Santa Clara, CA) in a GC17A gas chromatograph with a QP5000 MS spectrometer (Shimadzu Co., Columbia, MD) equipped with an autosampler (AOC-20, Shimadzu). The column temperature was programmed from 100°C for 10 minutes, then 3°C/min to 220°C, held for 5 minutes, followed by 40°C/min to 300°C and 300°C for 10 minutes. The injector and detector temperatures were set at 250°C and 280°C, respectively. Helium was used as the carrier gas at a head pressure of 50 kPa.

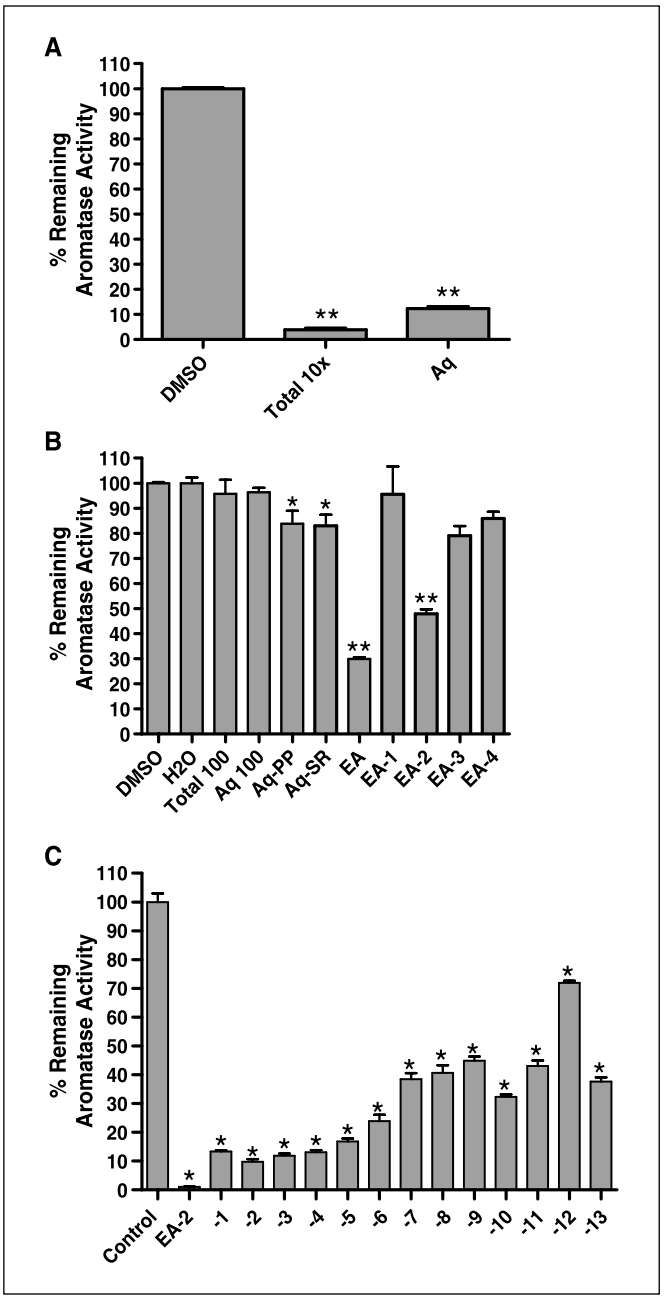

Figure 1. Inhibition of aromatase activity by white button mushroom extract and its fractions in a placental microsomal assay *in vitro*. Samples were treated with 10× mushroom extract (total 10×) and aqueous extract (Aq) mushroom (*A*), diluted extracts and their fractions at 100 mg/L (*B*), or EA2 fractions 1 to 13 (200 mg/L; *C*), and the aromatase inhibitory activity was quantified. *Total 10×,* 10×; *Total 100,* mushroom extract 100 mg/L; *Aq 100,* Aq extract 100 mg/L; *Aq-PP,* polysaccharide precipitate; *Aq-SR,* soluble residue. *Columns,* mean percentage of the activity of the vehicle control from triplicate analyses; *bars,* SE. *, $P \leq 0.05$; **, $P \leq 0.01$, compared with controls.

Analysis of fatty acid composition of mushroom extracts. Because fatty acids were found to be a major group of anti-aromatase chemicals in mushrooms, additional gas chromatography analyses were done to quantify the relative content of fatty acids in mushrooms. Fatty acids were extracted from 6× mushroom extracts using chloroform/methanol (2:1, v/v) with 0.2 vol. of 0.88% KCl (6). Fatty acid methyl esters of the fractions were prepared by incubating the fractions with tetramethylguanidine at 100°C (7) and analyzed by gas chromatography (Hewlett Packard) using a 30-m

Omegawax 320 (Supelco-Sigma, Bellefonte, PA) capillary column. The helium flow rate was 30 mL/min and oven temperature was ramped beginning at 175°C, held for 4 minutes, and then increased to 220°C at a rate of 3°C/min (8). Retention times were compared with authentic standards (Supelco-Sigma) and conjugated linoleate methyl esters (Matreya).

Placental microsome aromatase assay. The aromatase assay was done by modification of previous method (4). The substrate, [1-β-^{3}H]androste-H]androstenedione (specific activity, 25.3 Ci/mmol), was dissolved in serum-free cell culture medium. Placental microsomes were prepared as 0.1 g/L in a potassium phosphate buffer (67 mmol/L, pH 7.4) containing 20% glycerol, 0.5 mmol/L dothiothreitol, and 0.25 mol/L sucrose. The assay reaction mixture (225 μL), containing placental microsomes (2.5 μg), [^{3}H]androstenedione (50 nmol/L), progesterone (10 μmol/L), and bovine serum albumin (0.1%) in potassium phosphate (67 mmol/L, pH 7.4), with sample solution in DMSO or H_2O, was introduced in wells of a 96-well plate and preincubated at room temperature for 10 minutes; then, 25 μL of NADPH (3 mmol/L) were added and the mixture was incubated at 37°C for 15 minutes. The reaction was terminated by addition of 50 μL of 20% trichloroacetic acid, and 250 μL of the solution were transferred to another well containing the charcoal-dextran pellet. The solution was thoroughly mixed and centrifuged (1,000 × *g*, 5 minutes) to remove nonreacted substrate; an aliquot of the supernatant containing [^{3}H]H_2O as reaction product was counted in a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter. This modified assay method allows us to carry out 96 assays at the same time.

Aromatase inhibition activity was calculated as the percentage of remaining activity from the reaction without sample. Analyses were carried out in triplicate and data were expressed as the mean ± SE.

Enzyme inhibition kinetic analysis. The inhibition kinetics for linoleic acid, an inhibitor found in mushroom extract, were done with a range of 12.5 to 100 nmol/L of [1-β-^{3}H]androstenedione in triplicate. The procedure was the same as mentioned above and data were expressed as the mean ± SD.

"In-cell" aromatase assay. CHO cell lines that express the wild-type aromatase and its mutants have been prepared as previously described (9–11). These aromatase mutants have been shown to be valuable tools in evaluating the molecular basis of the interaction of aromatase inhibitors with aromatase (10) and in studying the reaction mechanism of the enzyme (11).

In the in-cell aromatase assay, aromatase-expressing CHO cells were plated on six-well plates in growth medium. When approximately confluent, the cells were washed twice with PBS and 1 mL of serum-free medium containing inhibitors at various concentrations along with 100 nmol/L [1β-^{3}H]androstenedione, as well as 1 μmol/L progesterone, used to suppress the endogenous 5α-reductase that also consumes the androgen substrate. The incubation was carried out for 1 hour, after which time the substrate mixture was removed and extracted with an equal volume of chloroform. The samples were then centrifuged at 1,000 × *g* for 10 minutes, and the aqueous upper layer from each sample was loaded onto a charcoal-dextran pellet to remove any trace of nonreacted substrate. The samples were vortexed and subsequently centrifuged at 15,000 × *g* for 5 minutes. Aliquots of the supernatant preparations were counted in a liquid scintillation counter. Aromatase activity was calculated as picomoles of tritiated water released per milligram of protein per hour. The cells in each well were solubilized in 0.5 N sodium hydroxide and the protein concentrations were quantified by the Bradford method (12).

The tritiated water release assay has previously been validated in our laboratory by the product isolation assay (13). In addition, the activities of the wild-type aromatase and its mutants have recently been evaluated by the reaction intermediate profile analysis along with the tritiated water release assay (11). Analyses were carried out in triplicate and data were expressed as the mean ± SE.

Cell proliferation studies. The estrogen receptor–positive, aromatase-overexpressing MCF-7 cell line MCF-7aro was prepared by stable transfection with the human placental aromatase gene and neomycin selection, as previously described (2). Aromatase activity in the transfected lines was determined to be 73 ± 6 pmol/mg/h. Experiments were initiated with 5×10^4 cells. For the first 3 days, cells were cultured with phenol red–free medium and 10% FBS, then in phenol red–free medium with 10% FBS that was pretreated with dextran-coated charcoal to remove any hormone-like activity. Culture medium was changed every 3 days. Proliferation-inducing agents (testosterone; Sigma Chemical) and test agents (6× mushroom extract, linoleic acid, or conjugated linoleic acid) were introduced into cultures on day 6. On days 7, 10, 13, and 16, cells were dissolved in 0.5 N NaOH. Total cell protein was determined by the Bradford method (3). The use of protein measurements for aromatase-mediated cell proliferation studies was previously confirmed by cell count analyses (14). We also examined the effect of mushroom extract on the growth of MCF-10A cell line (American Type Culture Collection, Bethesda, MD), a spontaneously immortalized normal epithelial cell line of mammary origin that does not produce tumors in athymic animals.

Animal experiments. Five- to 6-week-old female BALB/c *nu-nu*, athymic, ovariectomized mice were purchased from Charles River Laboratories (Wilmington, MA). At ~8 weeks of age, mice were s.c. implanted with 7.5 mg/60 day time-release androstenedione pellets (Innovative Research of America, Sarosota, FL). A week later, mice were individually gavage fed (orally force-fed) with 100 μL water control, or 100 μL of a 4× concentrated mushroom extract (in water). Each animal received daily gavage treatment for 42 consecutive days. At 10 weeks of age, mice were given two s.c. injections of MCF-7aro cells. These cells were grown in Eagle's MEM with nonessential amino acids, sodium pyruvate, and Earle's salts in 10% FCS. The MCF-7aro cells were harvested and resuspended in an equal volume of Matrigel (BD Biosciences, Bedford, MA) to a final concentration of 1×10^7 cells/0.2 mL. Body weights were monitored weekly as an indicator of the animal's overall health. At the end of 6 weeks of gavage treatment, mice were euthanized, blood samples were collected, and tumors and uteri were removed, weighed, and sent for H&E histologic staining to the City of Hope Pathology Department Core Facility. Tumor specimens were also stained with cleaved caspase-3 antibody for apoptosis and with Ki-67 antibody for cell proliferation.

Statistical analysis. To assess statistical significance, values were compared with controls by either Student's *t* test or one-way ANOVA, followed by Dunnett's multiple range test ($\alpha = 0.05$) using Prism GraphPad 4 software (GraphPad Software, Inc., San Diego, CA).

Results

Characterization of anti-aromatase chemicals in mushrooms. To identify potential anti-aromatase phytochemicals in mushrooms, the mushroom water extract (prepared according to

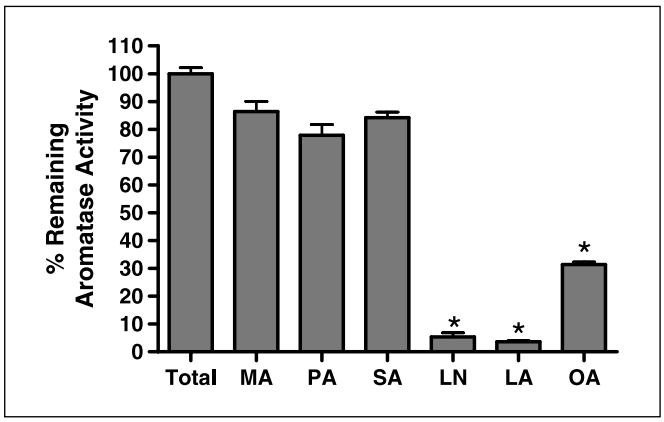

Figure 2. Inhibition of aromatase activity by fatty acids in a placental microsomal assay *in vitro*. Samples were treated with fatty acids (0.8 mmol/L) and the aromatase inhibitory activity was quantified. *Columns,* mean percentage of the activity of the vehicle control from triplicate analyses; *bars,* SD. *MA,* myristic acid; *PA,* palmitic acid; *SA,* stearic acid; *OA,* oleic acid; *LA,* linoleic acid; *LN,* linolenic acid. *, $P \leq 0.01$, compared with controls.

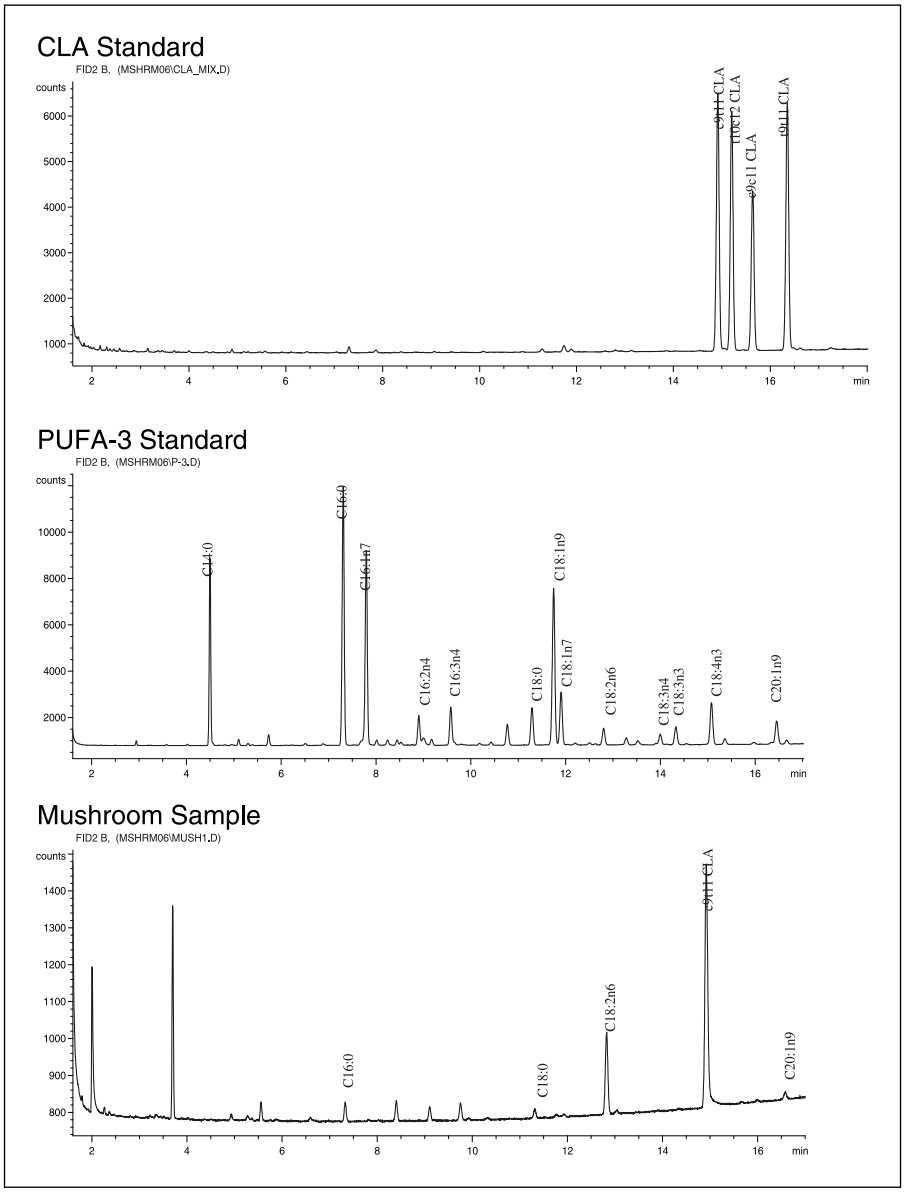

Figure 3. Identification of conjugated linoleic acid isomer c9t11 as a major fatty acid in mushroom extract. Procedures for the extraction and gas chromatography analysis of fatty acids are provided in Materials and Methods. Fatty acids were identified by comparing retention times of authentic standards and conjugated linoleate methyl esters.

procedures described in Materials and Methods) was partitioned with ethyl acetate to produce a water-soluble fraction [Aq; 98.5% of total extract (w/w)] and an organic-soluble fraction (EA; 1.5% of total extract). The aromatase inhibitory activities of Aq, EA, and their subfractions are shown in Fig. 1. The concentrated forms of the 10× mushroom and 10× Aq (5 g/L) were found to be potent inhibitors of aromatase activity (3.9 ± 0.72% and 12.3 ± 0.84% of total aromatase activity remaining, respectively; $P \leq 0.01$; Fig. 1*A*). However, when the 10× extracts were diluted to an equal concentration with respect to the isolated fractions (100 mg/L), the EA fractions proved to be more potent. Isolated from Aq, the 80% ethanol precipitates (polysaccharide precipitates) and soluble residues (low molecular sugars) showed weak inhibitory activity (remaining aromatase activity: Aq, 96.4 ± 1.77%; polysaccharide precipitate, 83.8 ± 5.11%; soluble residue, 83.0 ± 7.6% at 100 mg/L; $P \leq 0.05$; Fig. 1*B*). Both EA and EA2, a subfraction of EA, showed a potent aromatase inhibition at 100 mg/L (remaining aromatase activity: EA, 29.9 ± 0.64%; EA2, 43.9 ± 2.6% at 100 mg/L; $P \leq 0.01$; Fig. 1*B*). These results show that different phytochemicals in mushrooms exhibit anti-aromatase activity to varying degrees. In addition, we show that the organic-soluble constituents, which are only a small portion of the total mushroom extract, could be the most active anti-aromatase chemicals in mushrooms. Of the EA subfractions, EA2 likely contains the most active anti-aromatase phytochemicals.

The subfractions of EA2 (EA2-1–EA2-6) exhibited strong aromatase inhibitory activity (Fig. 1*C*). In GC-MS analyses, C-18 unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid, and conjugated linolenic acid) with saturated fatty acids [myristic acid (C-14), palmitic acid (C-16), and stearic acid (C-18)] were detected in these fractions. The other fractions, EA2-7 to EA2-13, in which fatty acids, simple organic acids (malic acid, lactic acid, and tartaric acid), and simple phenolic acids (benzoic acid, *p*-hydroxybenzoic acid, and α-hydroxybenzene propanoic acid) were found, showed weaker anti-aromatase activity (Fig. 1*C*) although the difference from controls was statistically significant for all

fractions tested ($P \leq 0.01$). These results suggest that the fatty acids found in EA2 are the more potent aromatase inhibitory constituents of mushrooms. Because these fatty acids are known to be ubiquitous compounds in food products, fatty acids were purified from mushroom with each of the compounds directly confirmed by comparisons of TLC chromatograms and GC-MS spectra with reference standards.

The fatty acids found in active fractions from the mushroom extract were evaluated for anti-aromatase inhibition activity (by human placental microsomal aromatase assay) using standard compounds. Among C-18 unsaturated fatty acids, linoleic acid (C18:2) exhibited most potent activity (IC_{50}, 0.21 mmol/L) followed by linolenic acid (C18:3) and oleic acid (C18:1; IC_{50}, 0.28 and 0.39 mmol/L, respectively; $P \leq 0.01$). The saturated fatty acids showed little inhibitory activity (remaining aromatase activity: myristic acid, 86.4 ± 3.7%; palmitic acid, 77.9 ± 3.8%; stearic acid, 84.2 ± 2.0% at 0.8 mmol/L; Fig. 2). These results indicate that C-18 unsaturated fatty acids are major aromatase inhibitors in organic soluble constituents of white button mushroom.

Because fatty acids were found to be a major group of anti-aromatase chemicals in mushrooms, additional gas chromatography analyses were done using 6× mushroom extracts (i.e., the extracts used for cell culture and animal studies) to quantify the relative content of fatty acids in mushrooms. The conjugated linoleic acid isomer c9t11 was identified in the active mushroom fractions (an average of 45.7% of extract; Fig. 3). Twenty-one percent of the total fatty acid content was linoleic acid. Experiments were then carried out to compare the potency of linoleic acid and conjugated linoleic acid in the inhibition of aromatase and in the modulation of proliferation of aromatase-positive breast cancer cells (i.e., MCF-7aro; discussed below).

Analysis of the interaction between aromatase and fatty acids found in mushroom. To determine the nature of the inhibitory mechanism of C-18 unsaturated fatty acids on aromatase, a double reciprocal plot of aromatase inhibition was generated with linoleic acid (Fig. 4). This analysis identified linoleic acid as a noncompetitive inhibitor of aromatase with respect to androstenedione, the substrate. Similar results were obtained when conjugated linoleic acid was tested (results not shown).

To characterize the interaction between linoleic acid and aromatase, we identified the amino acid residues important to this interaction using five aromatase mutants and the wild-type aromatase expressed in CHO cells (Fig. 5*A*). These mutants were chosen because, based on previous research in our laboratory (9–11), the mutated amino acid residues are thought to be key residues in the active site region of aromatase. The results indicate that His^{480} to Gln mutation and Ser^{478} to Thr mutation weakened the interaction of linoleic acid with aromatase. Alternatively, Glu^{302} to Asp mutation facilitated the interaction of this fatty acid with the enzyme.

Our results also showed that conjugated linoleic acid has similar potency and interacts with the aromatase mutants in an identical manner as linoleic acid (Fig. 5*B*). Therefore, conjugated linoleic acid and linoleic acid bind to aromatase in a similar fashion.

Cell proliferation studies. To determine the consequence of aromatase inhibition by mushroom extract in breast cancer cells, we did cell proliferation studies using the aromatase-overexpressing MCF-7aro cell line that was generated in our laboratory (14) and the MCF-10A nontumorigenic cell line. The MCF-7aro cell line is estrogen receptor positive/aromatase positive and shows increased cell proliferation in the presence of testosterone. Results showed that mushroom extract suppressed testosterone-induced proliferation of MCF-7aro cells in a dose- and time-dependent manner (Fig. 6*A*) whereas it was less effective against the proliferation of MCF-10A cells (Fig. 6*B*).

To further this observation, we tested the ability of linoleic acid and conjugated linoleic acid to inhibit the proliferation of tumorigenic, aromatase-overexpressing MCF-7aro cells. We found that conjugated linoleic acid inhibited the testosterone-dependent proliferation of MCF-7aro cells whereas linoleic acid had no effect (Fig. 6*C*). These results indicate that the physiologically relevant aromatase inhibitors in mushrooms are probably conjugated linoleic acid and its derivatives. We also examined the effect of linoleic acid and conjugated linoleic acid on the proliferation of MCF-10A cells. Because Majumder et al. (15) reported that conjugated linoleic acid could inhibit the proliferation of MCF-10A, a dose-response experiment was done. We found that under our cell culture conditions, the proliferation of MCF-10A cells was only slightly affected by either linoleic acid or conjugated linoleic acid.

Animal studies. To evaluate the breast cancer protective effects of white button mushroom *in vivo*, our laboratory investigated the ability of mushroom extract to inhibit MCF-7aro-derived tumor growth in athymic mice. The results of these animal experiments showed that the oral intake of mushroom extract significantly decreased tumor weight by 58% compared with control mice ($P \leq 0.01$; Fig. 7*A*). Histologic examination of the tumors (Fig. 7*D*) revealed that cell proliferation was significantly decreased by 21.6% in the mushroom group compared with controls ($P \leq 0.05$; Fig. 7*B*), whereas the levels of apoptosis between tumors from the control and mushroom extract–fed animals were similar (Fig. 7*C*). These data indicate that the tumor-suppressing effect of mushroom extract may be through inhibition of tumor cell proliferation and not through a cytotoxic effect.

Discussion

A variety of dietary substances, including plant extracts, fruits and vegetables, vitamins, minerals, and minor dietary constituents, are associated with a lower risk of breast cancer development (16–19). Published literature on the contribution of fruits and

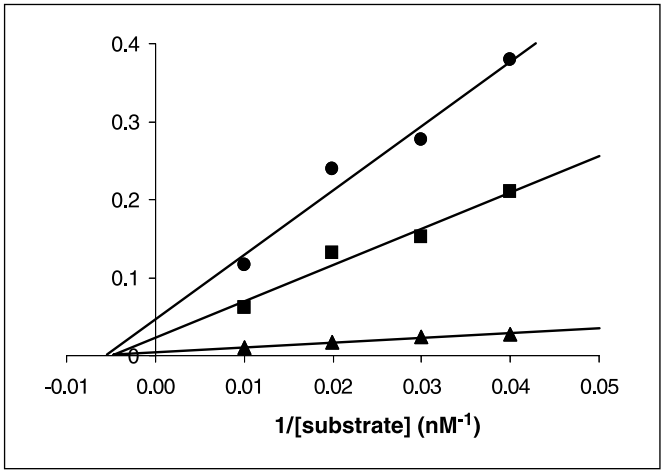

Figure 4. Kinetic analysis of androstenedione aromatization by linoleic acid in human placental microsomal assay. The assays were done with no inhibitor (▲) and with 150 μmol/L (■) and 300 μmol/L (●) linoleic acid.

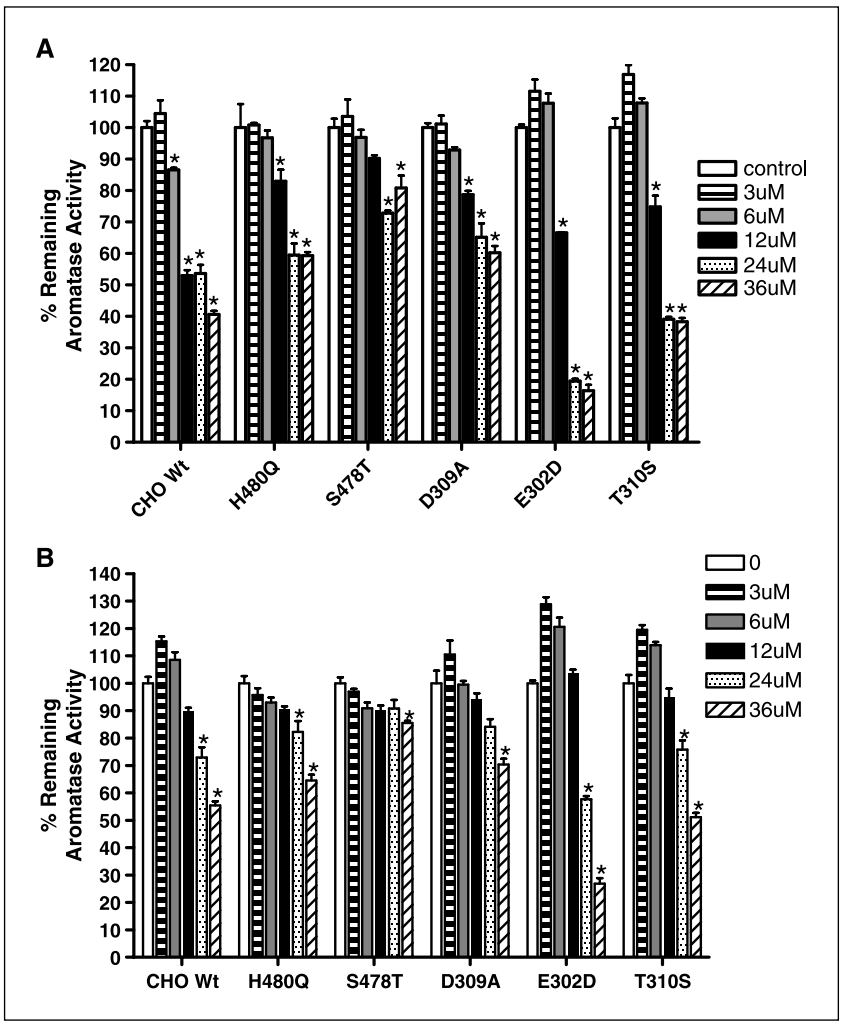

Figure 5. Effects of the mutations at the active site region of aromatase on the inhibitory activities of linoleic acid (*A*) and conjugated linoleic acid (*B*). In the presence of various concentrations of fatty acids, microsome assay was done on the wild-type and mutant human aromatase transfected CHO cells. The activities of the untreated samples were taken as 100%. *, $P \leq 0.01$, compared with untreated controls.

vegetables in relative risk reduction of breast cancer is conflicting and it is difficult to draw absolute conclusions from the data. However, a recent meta-analysis suggested an inverse relationship between intake of fruits and vegetables and the relative risk of developing breast cancer (20).

Medicinal mushrooms such as Maitaki have been reported to have anticancer properties. For example, the polysaccharide fraction of this mushroom extract, which was commercially developed as a dietary supplement for use as an immunostimulating agent and kidney tonic, was also suggested as an anticancer compound due to its immune-stimulating properties (21). To date, relatively few studies have been carried out on the white button mushroom. Some laboratories have reported potential anticancer effects of purified constituents of white button mushrooms such as the characterization of the volatile compounds (22), biological activities of 10-oxo-*trans*-8-decenoic acid (23), antiproliferative effect of lectin (24), and lignin degradation activity (25). Effects of vitamins, mineral elements, and simple phenolic acids have also been reported; however, the flavonoids (26–31) or lignans (32), which are known aromatase inhibitors, have not been detected in this and other laboratories. One report did suggest that the polysaccharides found in white button mushrooms may stimulate the immune system and inhibit tumorigenesis (33).

Results generated from this research project confirm our previous findings that white button mushrooms contain phytochemicals with anti-aromatase and anti-proliferative activities *in vitro*. Of the extracts tested, the organic-soluble compounds proved to be the most potent inhibitors of aromatase activity, with the fatty acid–rich EA2 fraction showing the most activity. The results also showed that proliferation was inhibited less in the MCF-10A noncancer cells than in the MCF-7aro cancer cells, suggesting that mushroom phytochemicals may exhibit selective inhibitory effects against breast cancer cells. The results further suggest that the unsaturated fatty acids, linoleic acid and conjugated linoleic acid, are relevant compounds in mushrooms. The water-soluble compounds (vitamins and mineral elements and carbohydrates) and simple phenolic compounds were not as effective at inhibiting aromatase activity *in vitro*.

Fatty acids are an integral component in living organisms and are known to have antioxidant and anti-inflammatory activities (34). From aromatase knockout and estrogen receptor knockout mouse studies, estrogen has been found to play an important role in the regulation of fatty acid–metabolizing enzymes (35–38). Consequently, a feedback mechanism involving fatty acids could be responsible for the down-regulation of aromatase expression or suppression of aromatase activity by mushrooms. In other studies, fatty acids and their derivatives have been shown to modulate

aromatase expression; however, the effect has mainly been positive (39, 40). Therefore, our finding that certain fatty acids can also act as inhibitors of aromatase is physiologically important. It is possible that differing effects of fatty acids on aromatase activity in cells provides a balance in the control of aromatase function.

Linoleic acid is an essential fatty acid in humans and is present in many foods including vegetables. Our results raise the question of how the unique anti-aromatase property of white button mushrooms may result from their linoleic acid and conjugated linoleic acid content. In other reports, linoleic acid induced breast cancer cell growth whereas conjugated linoleic acid suppressed breast cancer cell growth *in vitro* and *in vivo* (41, 42). Differential action of linoleic acid and conjugated linoleic acid on breast cancer cell proliferation was observed in our study. Both conjugated linoleic acid and linoleic acid inhibited aromatase activity; however, only conjugated linoleic acid showed antiproliferative activity. By gas chromatography analysis, conjugated linoleic acid was found to be a major fatty acid present in mushrooms. Taken together, these data lead us to conclude that the most physiologically important anti-aromatase phytochemical in mushrooms is conjugated linoleic acid or its derivatives. In addition to its action as an aromatase inhibitor, conjugated linoleic acid has been reported to modulate estrogen receptor function, leading to the suppression of proliferation in MCF-7 cells (43, 44). Liu and Sidell (45) have shown that conjugated linoleic acid can also modulate estrogen receptor phosphorylation and, therefore, activity. Furthermore, conjugated linoleic acid has been suggested to reduce cell proliferation by blocking DNA synthesis and cell cycle proteins that regulate this process (46, 47). It is known that linoleic acid can be converted to conjugated linoleic fatty acid by rumen bacteria (48). Therefore, it is possible that mushrooms may contain the proper enzymes or bacteria in the growth substrate to be capable of such conversion. Future studies in our laboratory will investigate this potential.

The nature of the interaction between aromatase and linoleic acid and conjugated linoleic acid proved to be noncompetitive in our experiments. The results from our aromatase studies in the aromatase mutant cells suggest that these fatty acids interact with aromatase at the active site region, with His^{480}, Ser^{478}, and Glu^{302} proving to be important to this interaction. The noncompetitive inhibition kinetic profile for these fatty acids implies that the substrate androstenedione cannot compete with them for their interaction at the aromatase active site. It is possible that these fatty acids may decrease the availability of binding site, possibly by blocking and/or altering the conformation of aromatase. This is another possible area of study for the future.

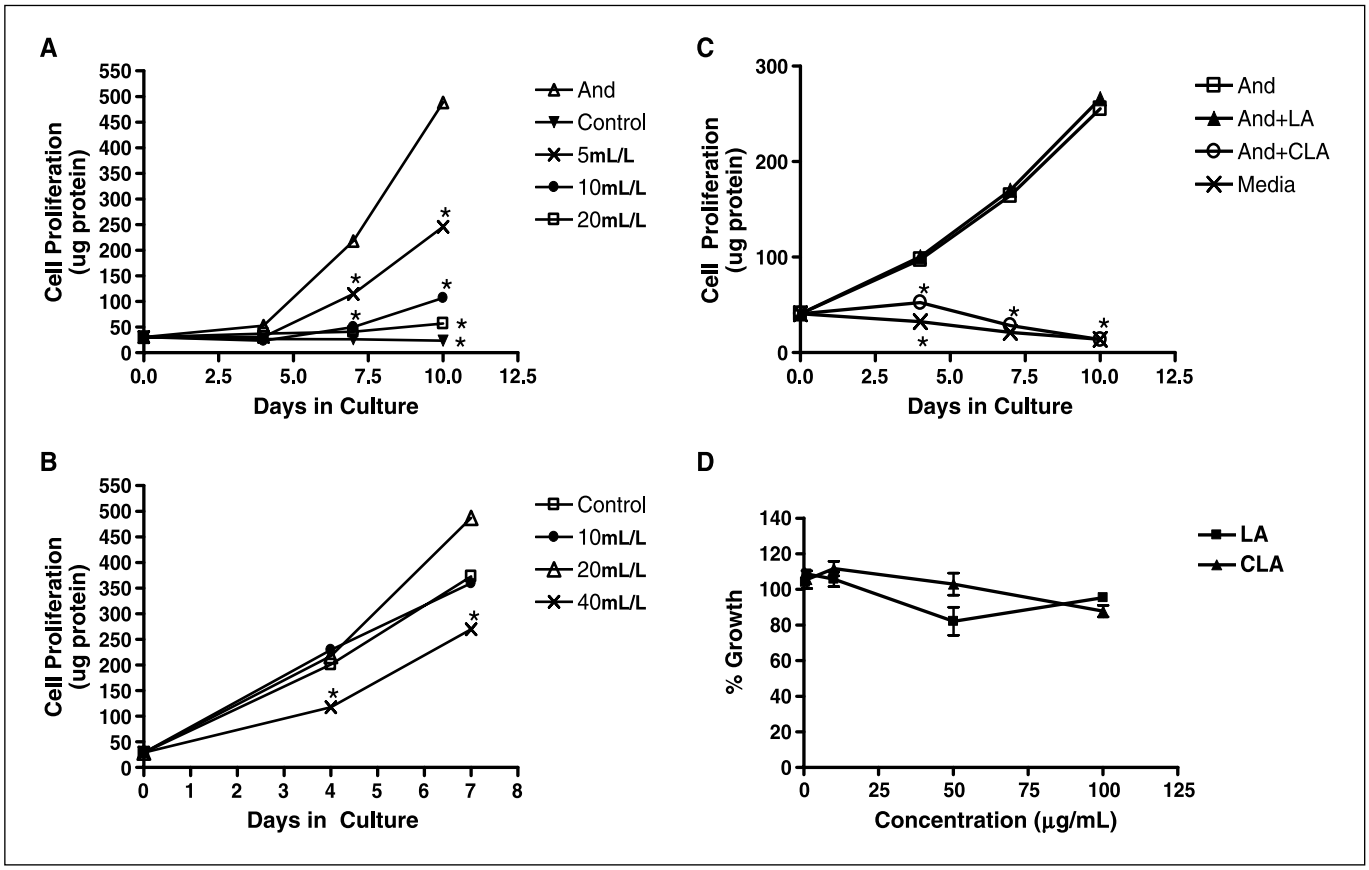

Figure 6. Effects of 6× white button mushroom extract and conjugated linoleic acid (*CLA*) on cell proliferation. *A,* MCF-7aro cells were cultured without androstenedione (▼), with 10 nmol/L androstenedione (△), or with 5 mL/L mushroom (×), 10 mL/L mushroom (●), or 20 mL/L mushroom (□) in the presence of 10 nmol/L androstenedione. *B,* MCF-10A cells were cultured without mushroom extract (□) or with 10 mL/L mushroom (●), 20 mL/L mushroom (△), or 40 mL/L mushroom (×). *C,* MCF-7aro cells cultured without androstenedione (×) or with 10 nmol/L androstenedione (□), 0.1 g/L linoleic acid (▲), or 0.1 g/L conjugated linoleic acid (○) in the presence of 10 nmol/L androstenedione. *D,* MCF-10A cells were cultured for 10 days with linoleic acid (■) or conjugated linoleic acid (▲) at different concentrations. Cells were cultured and protein concentration was determined as described in Materials and Methods. *Points,* mean of triplicate experiments; *bars,* SD. *, $P \leq 0.01$, compared with androgen-treated cells (*A* and *C*) and media control (*B*).

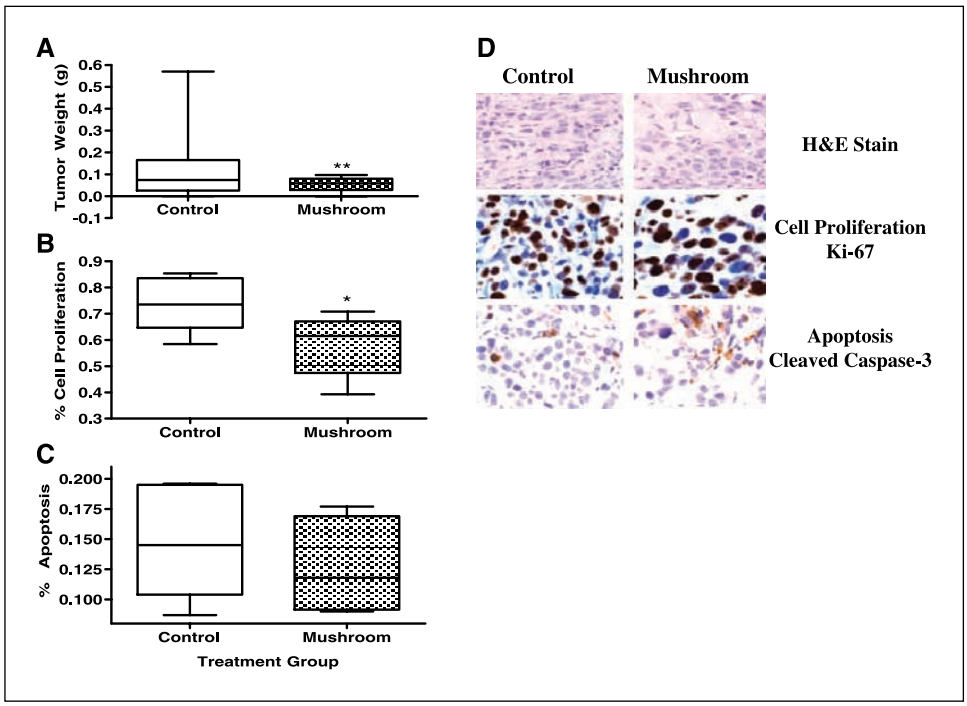

Figure 7. *In vivo* effect of mushroom extract on breast tumor growth. Seven- to 8-week-old athymic, nude, ovariectomized female mice were s.c. implanted with 7.5 mg/60 day time-release androstenedione pellets. The mice were gavage fed daily for 6 weeks with 100 μL of 4× mushroom extract or water. One week after implantation of androgen pellets, mice were given a 0.2-mL injection s.c. in each hind flank containing MCF-7aro cells mixed with equal volume of Matrigel. At the end of 6 weeks of gavage treatment, mice were euthanized; tumors were removed, weighed, and sent for histologic staining. Tumor weights (*A*), Ki-67 antibody staining for cell proliferation (*B*), and cleaved-caspase-3 antibody for apoptosis (*C*) were evaluated. *Points,* mean in each group; *bars,* SE. Statistics were done using the two-tailed Student's *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$, compared with control group.

Our *in vivo* studies illustrated that oral intake of mushroom extract suppressed the growth of hormone-dependent breast tumors in mice. These results indicate that the phytochemicals in mushroom, such as conjugated linoleic acid, are orally active and maintain their activity after ingestion. However, there is limited information available on the metabolic breakdown products of white button mushroom ingestion. Therefore, we plan to investigate this issue in future studies.

Our *in vivo* results also suggest that the phytochemicals in mushroom extract prevent tumor growth through the inhibition of cancer cell proliferation and not through the promotion of apoptosis. This suggests that further investigation into the molecular mechanisms of action of mushroom extract should focus on the proteins and signaling pathways integral to cancer cell proliferation.

Our analysis has found that unsaturated fatty acids are more potent inhibitors of aromatase than saturated fatty acids (Fig. 2). Furthermore, unsaturated fatty acids with more than one double bond (e.g., linoleic acid, conjugated linoleic acid, and linolenic acid) are more potent than those with only one double bond (e. g., oleic acid). These results are similar to those reported recently by Balunas et al. (49). The article by Balunas et al. also indicated that tested unsaturated fatty acids, including linoleic acid, were found to be inactive when tested with in-cell aromatase assay using SK-BR-3 cells. Based on their findings, Balunas et al. indicated that results from noncellular assays should be confirmed by in-cell assays. In our study, we have found that both conjugated linoleic acid and linoleic acid are effective in suppressing aromatase activity in CHO cells (Fig. 5) using in-cell assay. However, only conjugated linoleic acid is shown to effectively suppress the androgen-mediated proliferation of MCF-7aro cells. Therefore, after the demonstration of aromatase inhibition following enzyme assays, it is important to verify the action of inhibitors using cell proliferation bioassays.

As shown in Fig. 5, conjugated linoleic acid inhibits aromatase in CHO cells with an IC_{50} value of 12 μmol/L, which is much higher than those for known aromatase inhibitors (e.g., letrozole, anastrozole, and exemestane). These compounds inhibit aromatase with IC_{50} values between 1 and 10 nmol/L. It is clear that conjugated linoleic acid or mushrooms will not be very effective as therapeutic agents to treat breast cancer. However, consumption of conjugated linoleic acid or mushrooms may keep estrogen in postmenopausal women lower than normal, thus acting as potential preventive agents against hormone-dependent breast cancer. Using both 7,12-dimethylbenz(*a*)anthracene- and NMU-induced rat mammary tumor models, Ip et al. (50) have reported that conjugated linoleic acid has an antiangiogenic effect through the reduction of serum vascular endothelial growth factor (VEGF) and mammary gland VEGF and flk-1. It has also been reported that conjugated linoleic acid can up-regulate the cancer suppressor gene protein tyrosine phosphatase γ (*PTPγ*; ref. 51) and down-regulate prostaglandin E2 signaling (52). Prostaglandin E2, a major cyclooxygenase-2 product, has been shown to up-regulate the expression of aromatase in breast cancer cells. These results would suggest that conjugated linoleic acid could also suppress aromatase expression.

Results from this and other laboratories support the hypothesis that white button mushrooms may be an important dietary constituent for reducing the incidence of hormone-dependent breast cancer in women. Prevention strategies involving mushrooms are readily available, affordable, and acceptable to the general public. The dosage used in the *in vivo* trial is also physiologically relevant. A typical conversion factor for converting mouse to human dosage of chemotherapeutic agents is 25, calculated based on body surface area (53). Thus, according to our preliminary results generated from MCF-7aro-derived tumor formation in nude mice, consumption of 100 g of mushrooms per day would be sufficient to suppress breast tumor growth in women. As this is a pharmacologic dosage that inhibits the growth of established tumor cells *in vivo*, it is feasible that a lower intake of

mushroom ingested regularly as a routine dietary constituent would be effective in preventing the initiation of breast tumors in an average woman. The information gained from our study can aid in the design of more highly developed and effective breast cancer prevention strategies involving dietary constituents such as mushrooms.

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