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White Button Mushroom (*Agaricus Bisporus*) Exhibits Antiproliferative and Proapoptotic Properties and Inhibits Prostate Tumor Growth in Athymic Mice

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White button mushrooms are a widely consumed food containing phytochemicals beneficial to cancer prevention. The purpose of this research was to evaluate the effects of white button mushroom extract and its major component, conjugated linoleic acid (CLA) on prostate cancer cell lines in vitro and mushroom extract in vivo. In all cell lines tested, mushroom inhibited cell proliferation in a dose-dependent manner and induced apoptosis within 72 h of treatment. CLA inhibited proliferation in the prostate cancer cell lines in vitro. DU145 and PC3 prostate tumor size and tumor cell proliferation were decreased in nude mice treated with mushroom extract, whereas tumor cell apoptosis was increased compared to pair-fed controls. Microarray analysis of tumors identified significant changes in gene expression in the mushroom-fed mice as compared to controls. Gene network analysis identified alterations in networks involved in cell death, growth and proliferation, lipid metabolism, the TCA cycle and immune response. The data provided by this study illustrate the anticancer potential of phytochemicals in mushroom extract both in vitro and in vivo and supports the recommendation of white button mushroom as a dietary component that may aid in the prevention of prostate cancer in men.

BACKGROUND

The majority of common dietary constituents do not have an acute biological effect immediately after ingestion. However, when eaten daily over a lifetime, subtle, long-term effects may be observed. For this reason, cancer of the prostate, which requires many years to develop, is a prime target for prevention strategies utilizing daily factors such as dietary intake. It is estimated that the time required for the clonal outgrowth of a prostate cancer cell to develop into a 1 cm^3 primary prostate cancer would be 39.4 yr, with the mean age at diagnosis occurring at 72 yr of age (1). Epidemiologic studies have suggested that changes in lifestyle, including dietary modifications, could prevent a significant number of cancers (2–4). Of the environmental factors that affect prostate cancer development, diet has been identified as an important influence (5,6). Therefore, incorporating foods into the diet that are known to have chemopreventive properties could reduce cancer incidence and subsequently cancer-related deaths.

Several mushroom species such as *Ganoderma lucidum, Lentinus edodes, Grifola frondosa* and *Agaricus blazei* have been shown to exhibit anticancer effects such as the inhibition of cell proliferation in prostate (7,8), colon (9–11), and breast cancer cell lines (10,12). Mechanisms attributed to the anticancer activity of mushrooms include the induction of apoptosis; the inhibition of angiogenesis and prosurvival signaling pathways such as protein kinase B (AKT), extracellular-regulated kinase (ERK), nuclear factor kappa-B (NF6-B) and activating protein-1 (AP-1) (7,13,14) and modulation of the cell cycle control protein retinoblastoma (pRb) (8). Although much study has been completed on the medicinal varieties of mushroom mentioned above, studies into the anticancer effects of the common white button mushroom (*Agaricus bisporus*) are limited. Much of the research has focused on the anticancer effects of carbohydrate fractions of this species. For example, lectins isolated from the white button mushroom have been shown to increase the sensitivity of lung, colon, and glioblastoma cancer cells to chemotherapeutic drugs (15), inhibit colon cancer cell proliferation (16), and enhance cellular antioxidant defense mechanisms (17).

Previous studies in our laboratory investigated the antiaromatase activity of common vegetables that may suppress breast cancer cell proliferation. Of the 7 vegetable extracts tested, the extract of white button mushrooms was the most effective inhibitor of human placental aromatase activity (18). Our laboratory has focused on an ethyl acetate extract of the white button mushroom, which contains mainly C-18 fatty acids and simple organic and phenolic acids (19). We have previously reported that this extract effectively decreased the proliferation of breast cancer cells through the inhibition of aromatase enzyme activity.

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We also identified CLA as a major component of the mushroom extract and also an active inhibitor of breast cancer cell proliferation and aromatase activity (18,19).

To determine the anticancer potential of mushroom extract in prostate cancer cell lines, the current study investigated the in vitro effects of this extract on the androgen sensitive LNCaP and androgen insensitive PC3 and DU145 prostate cancer cell lines. We also studied the in vivo chemopreventive potential of mushroom extract in two separate studies utilizing male athymic mice injected with either DU145 or PC3 prostate cancer cell lines. The information gained from this study gives us future direction for investigation into the active ingredients of mushrooms as well as their mechanisms and to the overall understanding of how inclusion of mushrooms into the diet may contribute to more effective prostate cancer prevention strategies.

MATERIALS AND METHODS

Cell Culture

LNCaP, PC3, and DU145 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 containing 10% fetal bovine serum in the presence of 100 U/ml penicillin and 0.1 g/l streptomycin. Cells were incubated at 37°C with 95% air and 5% carbon dioxide. All cells were kept below passage 20 and used in experiments during the linear phase of growth.

Production of Mushroom Extract

Mushroom extract was produced by chopping 60 g of fresh white button mushroom (*Agaricus bisporus*) and boiling it in water. The broth was filtered and then applied to 5 g/60 ml capacity polyamide columns (Discovery DPA-6S SPE; Supelco, Bellefonte, PA). Fractions were eluted by a step gradient (50 ml of each step) of increasing methanol to water. The 20% methanol–water fraction was rotor evaporated to dryness and then redissolved in 1 ml of water to produce the 6× mushroom extract. Therefore, 6 g of mushroom can produce 100 μl of 6 × fraction.

Real-Time Proliferation Assay

Cells were plated in 16 well plates at a density of 10,000 cells/well and treated with either medium containing ethanol as vehicle control (<0.1% total) or CLA (Cayman Chemical, Ann Arbor, MI; 0–200 μM). Cell growth was monitored automatically via the ACEA RT-CES real-time proliferation machine (ACEA Biosciences, Inc., San Diego, CA), which swept the plates once an hour for up to 96 h. The machine measures electrical impedance through sensors on the bottom of the 16 well plates. Increase in impedance correlates with an increase in cell density. Media and treatments were changed after 48 h. Data are expressed as ratio of treated to untreated cells mean ± SE for 3 replications.

Apoptosis ELISA

Apoptosis was assessed utilizing the Cell Death Detection ELISA^{PLUS} Assay (Boehringer Mannheim, Indianapolis, IN). This assay is a photometric enzyme-linked immunoassay that quantitatively measures the internucleosomal degradation of DNA, which occurs during apoptosis. The assay is a quantitative sandwich-enzyme-immunoassay utilizing monoclonal mouse antibodies directed against DNA and histones that detect specifically mononucleosomes and oligonucleosomes. Quantitative measurement of the amount of internucleosomal degradation is measured photometrically at 405 nm with ABTS as substrate. Cells were plated in 60 mm dishes (Falcon, BD Biosciences, San Jose, CA) at a density of 100,000 cells/dish and allowed to attach for 24 h. Cells were treated with media control (mushroom extract is water soluble) or mushroom extract at a concentration of 20 μl/ml for 48 and 72 h. Following treatments, nonadherent cells were collected and pelleted at 200 *g* for 10 min. The supernatant was discarded; the cell pellet was washed with cold phosphate-buffered saline (PBS; 137 mmol/l sodium chloride, 1.5 mmol/l potassium phosphate, 7.2 mmol/l sodium phosphate, 2.7 mmol/l potassium chloride, pH 7.4) and recentrifuged. Adherent cells were washed with cold PBS, trypsinized, collected, and combined with nonadherent cells. Both live and dead cells were then counted via Trypan Blue (Pierce, Rockford, IL) exclusion, and 10,000 cells were added to the microtiter plate for all treatment groups; and apoptosis assay was performed according to the manufacturer's instructions. Absorbance was read on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. Background values were subtracted from readings (media plus reagent, no cells) and expressed as absorbance of dye bound to antibodies bound to mononucleosomes and oligonucleosomes at 405 nm of each treated sample divided by media controls.

Annexin V Assay

Cells were plated in 100 mm dishes (Falcon, BD Biosciences, San Jose, CA) at a density of 50,000 cells/dish and allowed to attach for 72 h. Cells were treated with media control (mushroom extract is water soluble) or mushroom extract at a concentration of 20 μl/ml for 48 and 72 h. Following treatments, adherent cells were trypsinized, nonadherent cells were collected, and all cells were pelleted at 200 *g* for 10 min and then washed twice with cold 1 × PBS and resuspended in 1 ml of 1 × binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. 1×10^5 cells were stained with Annexin V-FITC; BD Pharmingen, San Jose, CA) and propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) for 15 min at room temperature in the dark. After adding 400 ml of 1 × binding buffer to each tube, cells were analyzed by flow cytometry within 1 h on a CyAn^{TM} ADP 9color-UV flow cytometer(Dako, Inc., Carpinteria, CA). Controls included unstained cells, cells stained with Annexin V-FITC only, and cells stained with PI only.

Animal Experiments

Five-week-old, male BALB/c Nu-Nu, athymic mice were purchased (Charles River Laboratories). Mice were randomly divided into 6 groups with 8 mice per group. At 6 wk of age, mice were gavage fed with either 100 μl water control or 100 μl mushroom extract (in water). The third group was gavaged with 100 μl water and pair fed to the 6X mushroom group (the food consumed by the 6X mushroom group was weighed and the same amount provided to the pair-fed group) to control for differences in caloric intake. Each animal received daily gavage treatment for the duration of the experiment. At 7 wk of age, mice were given two subcutaneous injections of either DU145 or PC3 cells in Matrigel (BD Biosciences, San Jose, CA). These cells were grown in RPMI 1640 with nonessential amino acids, sodium pyruvate, and Earle's salts in 10% fetal calf serum. The cells were harvested and resuspended in an equal volume of Matrigel (BD Biosciences) 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 7 wk of gavage treatment, mice were euthanized; blood samples were collected; and tumors were removed, weighed, and sent for hematoxilin and eosin (H & E) histological staining through the City of Hope Pathology Department Core Facility. Tumor specimens were also stained using cleaved-caspase-3 antibody (Cell Signaling Technology, Danvers, MA) for apoptosis and Ki-67 antibody (Dakocytomation, Carpinteria, CA) staining for cell proliferation by the City of Hope Pathology Department Core Facility. Data are expressed as mean $\pm$ SEM ($n \geq 5$).

Microarray Analysis

For microarray analysis, total RNA was extracted from 3 DU145-derived tumors from each treatment group using TRIzol reagent (Invitrogen, Carlsbad, CA). Synthesis and labeling of cRNA targets, hybridization of GeneChips, and signal detection were carried out by the Microarray Core Facility at the City of Hope. Briefly, biotinylated cRNA was generated using 5 μg total RNA using T7 RNA polymerase. The Affymetrix GeneChip Human Genome U133A v2.0 array (HGU133A2) (Affymetrix, Santa Clara, CA) was used to define gene expression profiles from tumor samples. For microarray hybridization, the GeneChip arrays were hybridized with 15 μg of fragmented cRNA targets and then washed. The staining was performed with streptavidin-PE. Affymetrix GeneChip images were scanned at 11-μm resolution using a high resolution GeneChip Scanner 3000 (Hewlett-Packard).

Statistical Processing of Microarray Data

Quality assessment and statistical analysis of gene expression data was performed using the R/Bioconductor packages. To ensure the high quality of the microarray process, a set of quality assessment steps implemented in Bioconductor package "AffyExpress" were applied to the data. Raw intensity measurements of all probe sets were converted into expression measurements using the "GCRMA" package. The "LIMMA" package was then used to identify the genes differentially expressed between mushroom-fed and water-fed samples. The genes showing altered expression were categorized on the basis of their cellular components, biological processes, molecular functions, and signal pathways using the Ingenuity Pathways Analysis (Ingenuity, Mountain View, CA) software. Significant genes were selected with a cutoff of $P < 0.01$ and log2 ratio of 1 (twofold change).

Ingenuity Pathway Analysis (IPA)

IPA is a Web-based software program that identifies the biological functions, pathways, and mechanisms most relevant to a given data set of genes. Information on individual genes is drawn from a large knowledge base of biological networks created from millions of publications (full-text articles published in scientific journals), and the networks are drawn by the Functional Analysis feature of IPA based on the connectivity of the genes.

Real-Time PCR

Trizol reagent (Invitrogen) was used for total RNA isolation. SYBR Green Supermix and iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) were used for cDNA preparation. PCR primers for KIT were as follows: 5′GCCGACAAAAGGAGATCTGT3′ and 5′CCTTTGCCACCTGGTAAGAA3′; for FH, 5′CCGCTG-AAGTAAACCAGGAT3′ and 5′TCCTGATCCAGTCTGCCATA3′; for FAS, 5′ATC-AAGGAATGCACACTCACC3′ and 5′GGTTGGAGATTCAT-GAGAACC3′; for human β-actin (used as an internal control), 5′AGAAGGAGATCACTGCCC-TGGCACC3′ and 5′CCTGCTTCGTGATCC-ACATCTGCTG3′. Reactions were run in triplicate on the iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and results were analyzed with the software provided.

Statistical Analysis

To assess statistical significance, values were compared to controls with either Student's t-test or 1-way analysis of variance (ANOVA), followed by Dunnett's Multiple Range test ($\alpha = 0.05$) or 2-way ANOVA as appropriate using Prism GraphPad 4 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Effects of Mushroom Extract on Prostate Cancer Cell Proliferation

The antiproliferative activity of mushroom extract was assessed in the LNCaP, DU145, and PC3 prostate cancer cell lines. Cells were treated with mushroom extract (20 μl/ml) for 96 h. Results from this assay showed that exposure to mushroom extract resulted in a significant, dose-dependent inhibition of cell proliferation ($P \leq 0.01$) in all cell lines tested. The magnitude

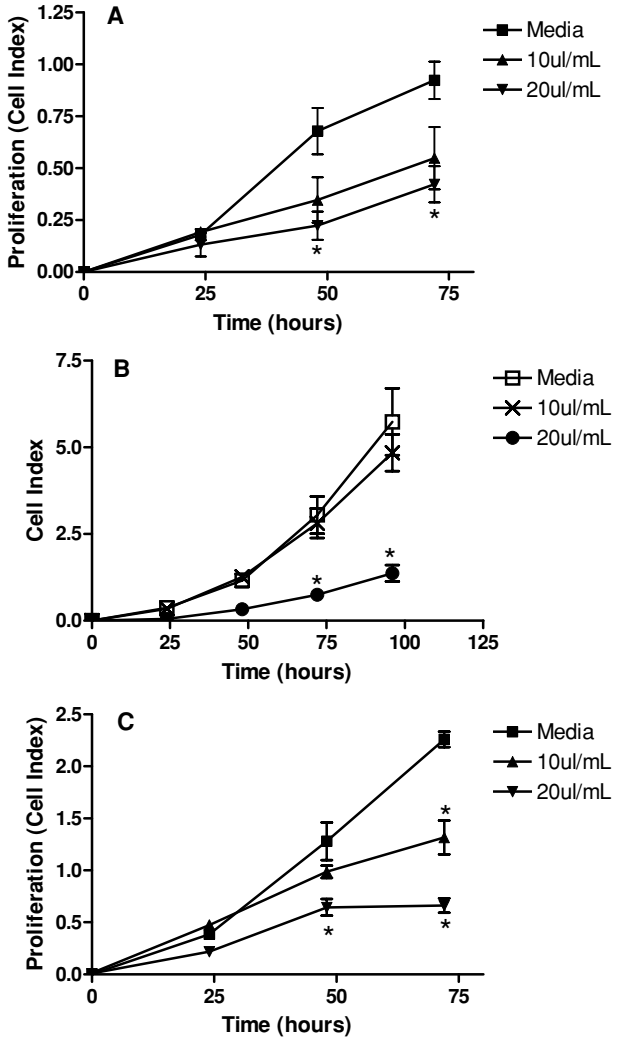

FIG. 1. Antiproliferative activity of mushroom extract on prostate cell lines. LNCaP (A), DU145 (B), and PC3 (C) cells were plated in the ACEA 16-well plate (5,000 cells/well) in media alone or treated with indicated concentrations of mushroom extract. Readings were taken every hour up to 96 h by the ACEA machine and expressed as cell index (a measure of cell number/well). Data represent means ± SE ($n = 3$); * indicates significant difference from media control ($P \leq 0.01$). LNCap, lymph node carcinoma of the prostate.

of response to mushroom extract was similar between cell lines (Fig. 1).

Induction of Apoptosis by Mushroom Extract

To determine whether the observed decrease in cell number after treatment with mushroom extract was due to the induction of apoptosis, the formation of mononucleosomes and oligonucleosomes was quantified using the Cell Death Detection ELISA^{PLUS} assay (Roche Diagnostics, Mannheim, Germany) and Annexin V/PI staining. Following treatment with mushroom extract (20μl/ml) for 48 h, a significant twofold increase in DNA fragmentation was detected in the LNCaP cells ($P \leq 0.01$) compared to untreated controls (Fig. 2A). The PC3 and DU145 cell

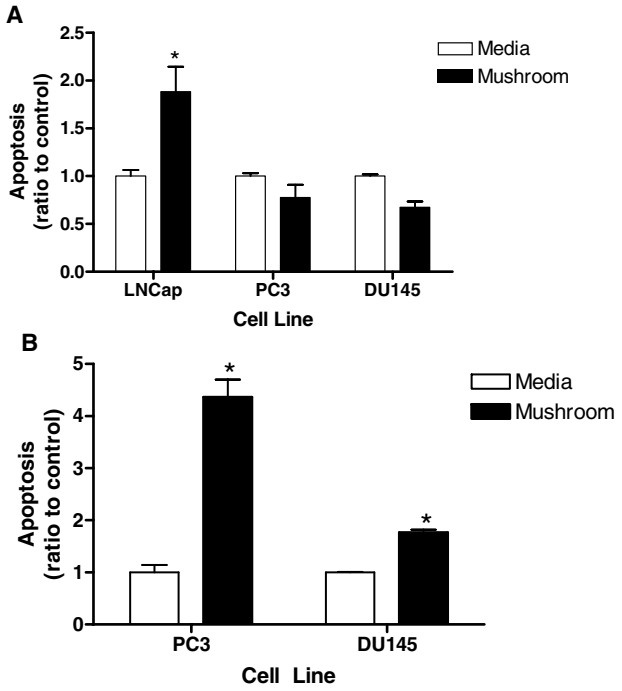

FIG. 2. Mushroom extract induces DNA fragmentation in prostate cell lines. Cells were exposed to mushroom extract (20 μl/ml) for 48 h (A) or 72 h (B), harvested, and analyzed using the Cell Death Detection ^{PLUS} Assay. Values are means ± SE, $n = 3$. Data are expressed as absorbance at 405 nm of each treated sample divided by vehicle controls. * indicates a significant difference ($P \leq 0.001$) compared to untreated controls.

lines showed no increase in the production of mononucleosomes and oligonucleosomes with mushroom treatment compared to untreated controls at 48 h (Fig. 2A). However, after 72 h of treatment, mushroom extract significantly induced DNA fragmentation 4.4-fold in the PC3 cells ($P \leq 0.001$) and 1.8-fold in the DU145 cells ($P \leq 0.001$; Fig. 2B).

The translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane was determined using Annexin V-FITC/PI staining and flow cytometry. Changes in the location of cell surface markers, such as PS, are the earliest detectable features of apoptotic cells. As such, PS detection in intact cells (PI negative cells) represent an early stage of apoptosis in the cell lines tested following exposure to mushroom extract. In the LNCaP cells, Annexin V positive cells increased 4.4-fold with mushroom treatment after 48 h compared to untreated controls ($P = 0.025$). In PC3 cells treated with mushroom extract, Annexin V staining increased threefold ($P \leq 0.01$), whereas the DU145 cells exhibited a 1.4-fold increase compared to untreated controls (Fig. 3A). After 72 h of treatment with mushroom extract, PC3 cells had a twofold increase in Annexin V staining ($P \leq 0.02$), whereas DU145 cells had a 1.6-fold increase in Annexin V staining ($P \leq 0.02$; Fig. 3B). These results illustrate that the LNCaP cell line is more sensitive to the proapoptotic effects of mushroom extract compared to the

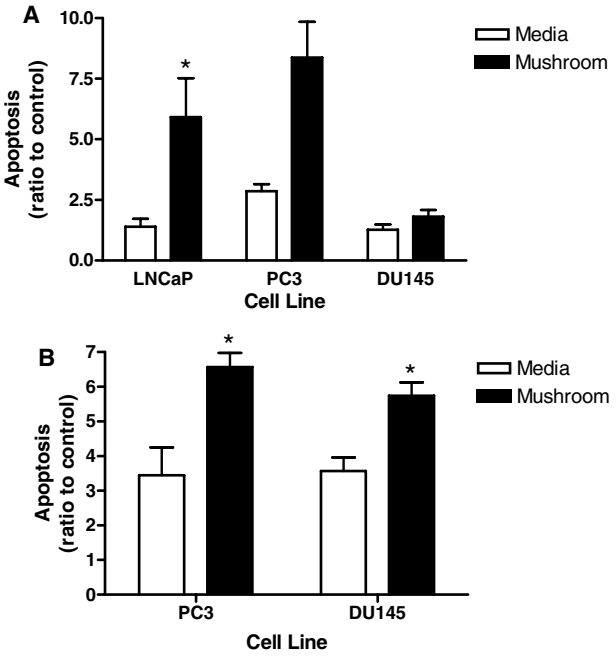

FIG. 3. Detection of phosphatidylserine externalization in prostate cells treated with mushroom extract. LNCaP, PC3, and DU145 cells were exposed to mushroom extract (20 μl/ml) for 48 h (A) and DU145 and PC3 cells were exposed to mushroom extract (20 μl/ml) for 72 h (B). Cells were stained with Annexin V-FITC and PI and analyzed on a FACS calibur flow cytometer. Data are expressed as percentage of Annexin V positive cells divided by Annexin V negative cells, mean $\pm$ SE, $n = 3$. * indicates a significant difference ($P \leq 0.05$) compared to untreated controls.

PC3 and DU145 cell lines, which respond at a later time point. In addition, the DU145 cell line showed only a mild response to mushroom extract and is therefore more resistant than the other two cell lines. Additionally, this shows that the antiproliferative activity is distinct from the proapoptotic activity of mushroom, as all three cell lines responded equally in the proliferation assay.

Inhibition of Tumor Growth In Vivo

To evaluate the prostate cancer protective effects of white button mushroom in vivo, our laboratory investigated the ability of mushroom extract to inhibit PC3 and DU145-derived tumor growth in male, athymic (nu/nu) mice. The results of these animal experiments showed that the oral intake of mushroom extract significantly decreased DU145 tumor weight 44.5% ($P \leq 0.05$) (Fig. 4A) and PC3 tumor weight 68.6% ($P \leq 0.01$; Fig. 5A) compared to pair-fed control mice. There was no difference in mouse weight between treatment groups. Histological examination of DU145 tumors revealed that cell proliferation was significantly decreased 25.3% in the mushroom group compared to controls ($P \leq 0.05$; Fig. 4B), whereas the level of apoptosis between tumors from the control and mushroom extract-fed animals was increased, although this observation was not statistically significant (Fig. 5B). Histological examination of the PC3 tumors showed that cell proliferation was significantly decreased 45% in the mushroom group compared to controls ($P \leq 0.01$; Fig. 4C), and the level of apoptosis increased 200% ($P \leq 0.001$; Fig. 5C). The LNCaP cell line did not reliably form tumors when implanted into the athymic mice, therefore we chose not to utilize that cell line for this study. The DU145 and PC3 data illustrates the ability of mushroom extract to decrease the growth and induce apoptosis of prostate cancer cells in vivo.

Inhibition of Prostate Cell Proliferation by Conjugated Linoleic Acid

Through GC analysis, we previously identified conjugated linoleic acid isomer 9Z, 11E as a major component in the 6X mushroom fraction (an average of 45.7% of the total extract). We also showed that 9Z, 11E CLA alone inhibits breast cancer cell proliferation through inhibition of the activity of the aromatase enzyme (19). Therefore, we tested the ability of the two common isoforms of CLA (9Z, 11E and 10E, 12Z) to inhibit prostate cancer cell proliferation. The results indicate that both isoforms of CLA significantly inhibited prostate cancer cell proliferation in a dose dependent manner (9Z, 11E with a P value $\leq$0.01 and 10E, 12Z with a P value $\leq$0.05; Figs. 6A–6F), suggesting that CLA may be one of the major active components in mushroom extract. However, in natural products, it is generally accepted that the overall combination of components can be more potent than any one component singled out. Further, it is possible that one of the minor components could be as active as CLA; however, in our breast cancer studies, the ethyl acetate fraction was the most potent fraction, and the major constituent was found to be CLA.

Validation of Microarray Results by Real-Time PCR

Microarray analysis results were validated by real-time PCR using primers for three of the most highly regulated and interesting genes (KIT, FAS, and FH) in tumor samples. Results showed a significant decrease in KIT expression and significant increases in the expression of FAS and FH (Figs. 7A–7C), which is in agreement with the microarray analysis results.

Network Analysis of Mushroom Responsive Genes

Genes that were regulated by mushroom extract in DU-145 tumor samples were analyzed for their functional grouping using IPA. Six networks of genes were significantly altered by mushroom extract identified by IPA in our data set (Table 1). Of these networks, 2 were of prime interest to this investigation: those grouping genes important to cell death, cellular growth and proliferation, and cellular morphology and those grouping genes important to cellular growth and proliferation, lipid metabolism, and small molecule biochemistry. An illustration of these gene networks can be found as supplemental Data 1 and 2, respectively.

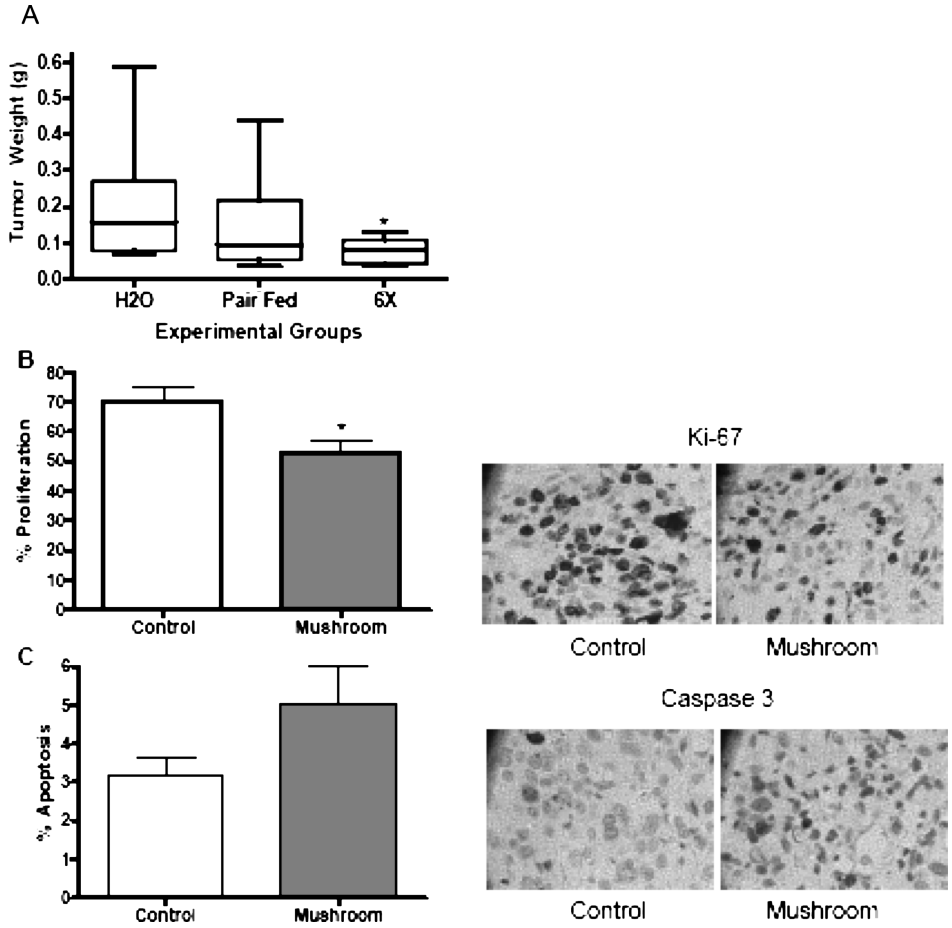

FIG. 4. In vivo effect of mushroom extract on DU145 prostate tumor growth. 6-wk–old, athymic, nude male mice were gavage fed with either 100 μl water control or 100 μl mushroom extract (in water). Each animal received daily gavage treatment for 7 wk. After 1 wk of gavaging, mice were given 2 subcutaneous injections of DU145 cells in Matrigel (BD Biosciences, San Jose, CA). 6 wk after injection, mice were euthanized and A: tumor weights, B: Ki-67 antibody staining for cell proliferation, and C: cleaved-caspase-3 antibody for apoptosis were evaluated. Data represent mean in each group ($n \geq 5 \pm$ standard error of the mean). * indicates statistical significance from control group ($P \leq 0.05$).

Within the gene networks, specific biological functions such as apoptosis, lipid metabolism, and immune response were altered by mushroom extract. These groupings suggest possible mechanisms of action for the effects of mushroom extract observed in the prostate tumors. Table 2 shows the gene symbol, Entrez Gene number, fold change from controls, and description of these gene groupings.

DISCUSSION

The aim of this research was to test the anticancer activity of phytochemicals in the common white button mushroom (*Agaricus bisporus*) in prostate cancer in vitro and in vivo. Our results show that mushroom extract decreased the proliferation of the prostate cancer cell lines in a dose-dependent manner compared to untreated control cells. It is important to note that mushroom extract had a similar antiproliferative effect on all three cell lines despite their differing status with regard to androgen responsiveness. Therefore, we can conclude that the antiproliferative action of mushroom extract is mediated through an androgen-independent mechanism.

As seen in Figs. 2 and 3, differing levels of sensitivity to apoptosis induced by mushroom extract exist between the three cell lines, with the LNCaP being the most sensitive, the PC3 less sensitive, and the DU145 the least responsive to the proapoptotic effects of mushroom extract. A similar result was seen in a study utilizing an extract of *Phellinus linteus* (PL) mushroom in which LNCaP cells underwent apoptosis to a larger degree than PC3 cells after treatment with PL. This study showed that caspase 2 expression was upregulated in LNCaP cells but not PC3 cells, and inhibition of caspase 2 abolished the proapoptotic effect of PL in LNCaP cells. The proapoptotic proteins caspase 8, caspase 3, and BID were activated equally in both LNCaP and PC3 cells (20), suggesting that although apoptosis may be induced along similar pathways in DU-45, PC3, and LNCaP prostate cancer cell lines, the modulation of additional pathways in androgen

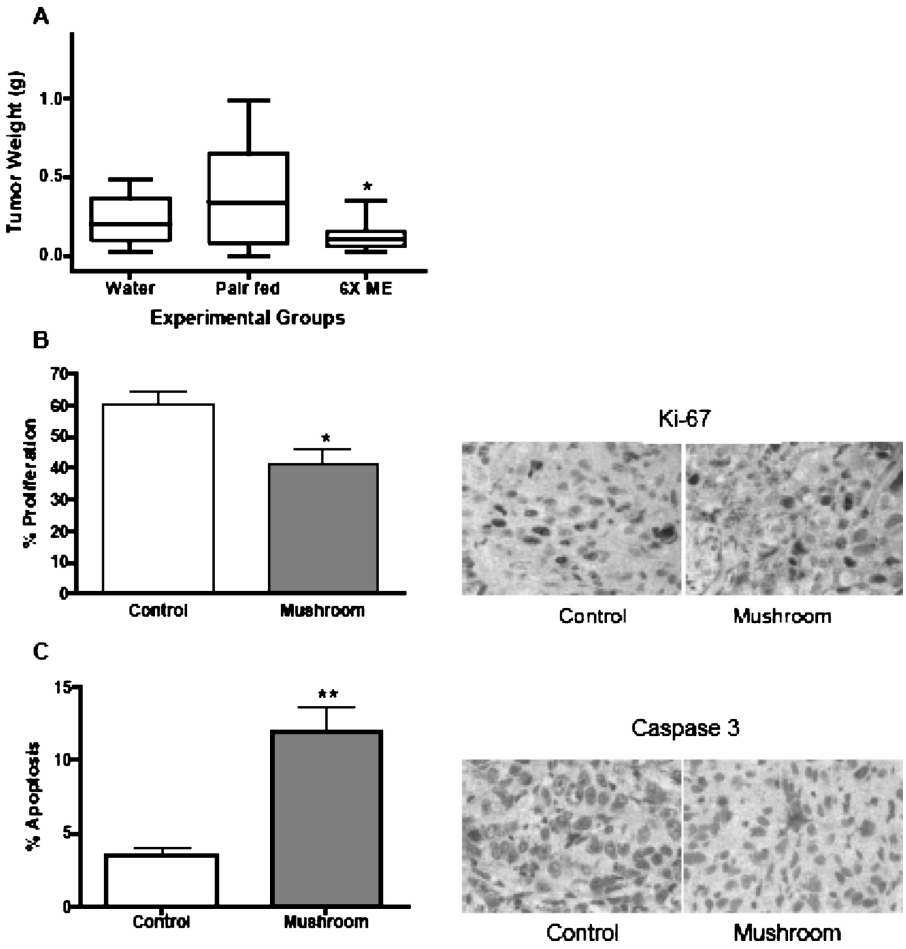

FIG. 5. In vivo effect of mushroom extract on PC3 prostate tumor growth. 6-wk–old, athymic, nude male mice were gavage fed with either 100 μl water control or 100 μl mushroom extract (in water). Each animal received daily gavage treatment for 7 wk. After 1 wk of gavaging, mice were given 2 subcutaneous injections of PC3 cells in Matrigel (BD Biosciences, San Jose, CA). 6 wk after injection, mice were euthanized and A: tumor weights, B: Ki-67 antibody staining for cell proliferation, and C: cleaved-caspase-3 antibody for apoptosis were evaluated. Data represent mean in each group ($n \geq 5 \pm$ standard error of the mean). * indicates statistical significance from control group ($P \leq 0.01$); ** indicates statistical significance from control group ($P \leq 0.001$).

responsive, LNCaP cells may sensitize them further and make them more susceptible to apoptosis. Future studies will be done to determine the mechanisms behind the differing response from each cell line.

Our in vivo studies illustrated that oral intake of mushroom extract suppresses the growth of androgen-independent prostate tumors in mice. This shows that the phytochemicals present in mushroom are active after oral ingestion. Further, the suppression of growth was due to inhibition of cell proliferation as measured by Ki-67 antibody staining, and induction of apoptosis was observed. This result is in agreement with the in vitro results. Our previous study (19) did not show apoptosis as a mechanism of mushroom extract in breast cancer cells lines and tumors, whereas in the current study in prostate cancer, apoptosis was observed in the prostate cell lines. It is well known that distinct cell lines within the same cancer family can respond differently to treatment (as was seen in the current study); therefore, it is reasonable to expect that results in two different cancer types could also be dissimilar.

Results from microarray analysis suggest several mechanisms for the effect of mushroom on prostate cancer cell proliferation and apoptosis. Although the expression of many genes was affected, two genes of primary interest are FAS/APO-1 and KIT. FAS/APO-1 is a member of the tumor necrosis factor receptor superfamily. This receptor contains a death domain and plays a central role in the physiological regulation of programmed cell death (apoptosis). This gene was upregulated 2.84-fold in tumors from the mushroom group compared to pair-fed controls ($P = 0.035$). KIT encodes the human homolog of the proto-oncogene c-kit, and the protein product plays a role in the proliferation and survival of the cell. KIT gene expression was downregulated fourfold in tumors from the mushroom group compared to pair-fed controls ($P = 0.043$). These results suggest that mushroom extract may have a favorable effect on

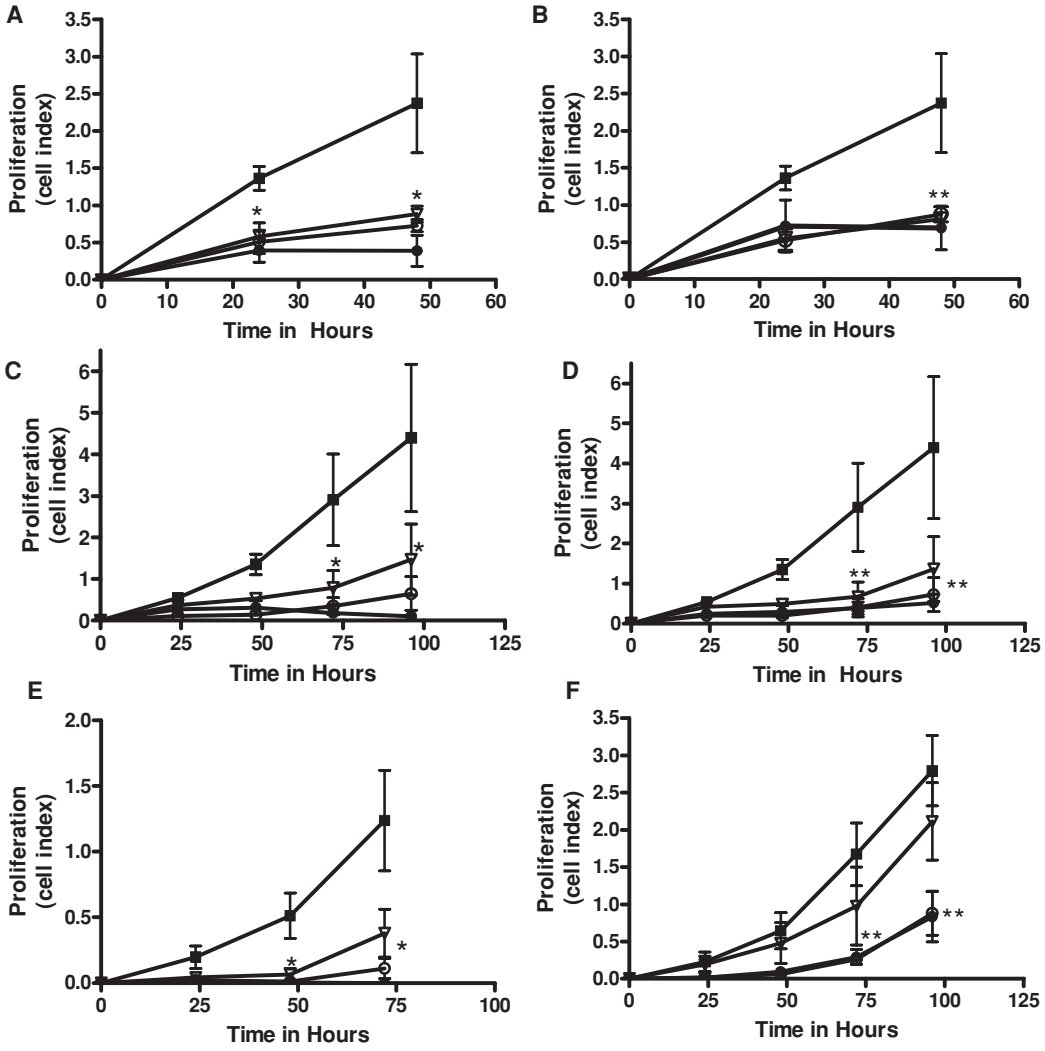

FIG. 6. CLA inhibits proliferation of prostate cancer cell lines. Cells were plated in the ACEA 16-well plate (5,000 cells/well) in media alone or treated with indicated concentrations of CLA; A: LNCaP, 9Z, 11E CLA; B: LNCaP, 10E, 12Z CLA; C: DU145 9Z, 11E CLA; D: DU145, 10E, 12Z CLA; E: PC3, 9Z, 11E CLA; F: PC3 10E, 12Z CLA. Readings were taken every hour up to 96 h by the ACEA machine and expressed as cell index (a measure of cell number/well). Data represent means ± SE ($n = 3$). * indicates significant difference from media control ($P \leq 0.01$); **indicates significant difference from media control ($P \leq 0.05$). Symbols: ■, vehicle; ∇, 50 μM CLA; ○, 100 μM CLA; ●, 200 μM CLA.

the proliferation and survival of prostate cancer cells in vivo through increased expression of FAS and decreased expression of KIT.

Microarray analysis results also indicate that the net effect of mushroom extract on lipid metabolism in tumor tissues could result in a decrease of diacylglycerol (DAG) and prostaglandin E2 (PGE_2) production/release while increasing ceramide and arachidonic acid release. Both ceramide and arachidonic acid are involved in proapoptotic signaling (21–23) in cancer cells. DAG production leads to the activation of PKC and the subsequent inhibition of ceramide-mediated apoptosis (24). Therefore, mushroom could also increase apoptosis through increased production of arachidonic acid (an eicosanoid) and ceramide, which is facilitated by inhibition of DAG production. Additionally, a study by Ochoa et al. (25) found that the 9Z, 11E isomer of CLA, which was found to be prominent in mushroom extract, had significant effects on cell proliferation and apoptosis through modulation of eicosanoid biosynthesis in PC3 prostate cancer cells. Specifically, cyclooxygenase-2 (COX-2) protein expression and 5-lipoxygenase (5-LOX) mRNA expression were both decreased in cells treated with 9Z, 11E CLA, suggesting that modulation of eicosanoid biosynthesis may be one mechanism by which CLA inhibits prostate cell proliferation (25). This data is in agreement with the observed effects in the current study of CLA-rich mushroom extract in prostate cancer cells.

Arachidonic acid is converted to PGE_2 through the (COX-2) pathway, and PGE_2 production is commonly increased in tumor tissues. PGE_2 itself has been shown to promote

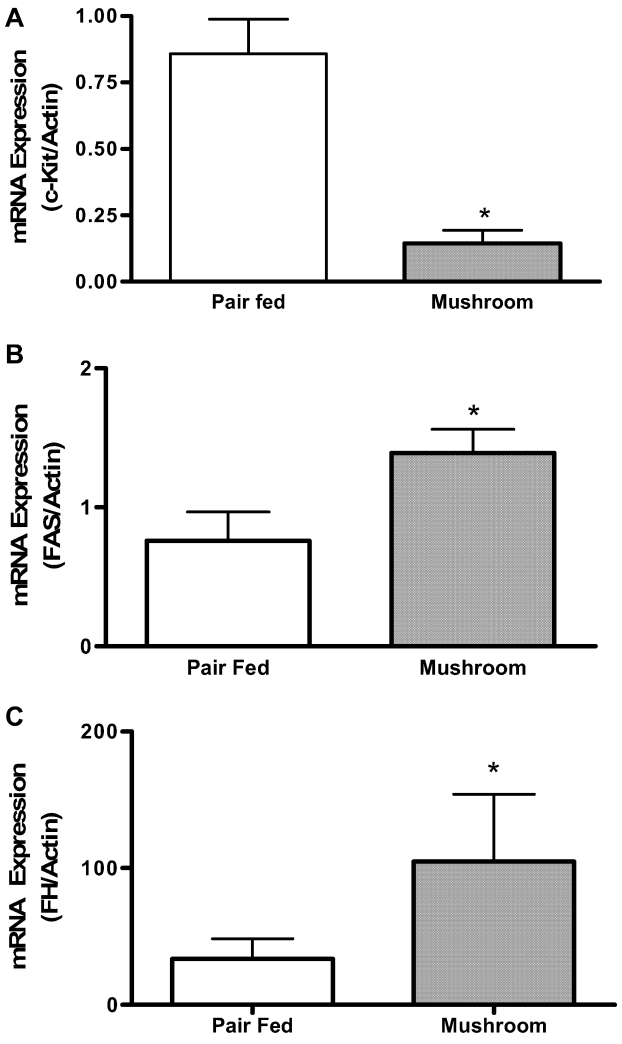

FIG. 7. Relative expression levels of c-KIT, FAS, and FH in tumor samples. mRNA expression of A: c-Kit, B: FAS, and C: FH in tumor samples was determined by quantitative, real-time PCR, and the expression of β-actin was monitored as the internal control. Data represent means $\pm$ SE ($n = 3$); * indicates significant difference from pair-fed control ($P \leq 0.01$).

cancer progression by increasing angiogenesis through vascular endothelial growth factor (VEGF) activation, blocking apoptosis through activation of the phosphatidylinositor 3-kinase (PI3K)/AKT/peroxisome proliferator-activated receptor (PPAR) signaling pathway, increasing tumor cell proliferation through activation of the RAS/RAF/MEK/ERK signaling pathway and affecting immune suppression by increasing interleukin 10 (IL10) production (23,26). Therefore, the suppression of PGE_2 is one way in which mushroom extract may inhibit prostate tumor cell growth in vivo.

These effects on lipid metabolism are complimented by the inhibition of isocitrate dehydrogenase 2 (IDH2) and increased expression of FH, 2 key tricarboxylic acid (TCA) cycle proteins, which have also been shown to play a role in fatty acid metabolism and tumor angiogenesis. IDH2 expression was downregulated threefold ($P = 0.017$), whereas FH expression was upregulated 4.3-fold ($P = 0.009$) in the mushroom-treated group compared to pair-fed controls. This is of interest due to the unique nature of normal prostate epithelial cells to secrete citrate rather than utilize it in the TCA cycle for the production of ATP. Prostate cancer cells, however, alter this balance by upregulating the citric acid cycle to produce more energy through increased ATP production (27). Therefore, mushroom extract may normalize the TCA cycle in prostate cancer cells by downregulation of isocitrate dehydrogenase expression. Citrate is converted to acetyl co-A, which is an instrumental component in fatty acid synthesis, leading back to the above-mentioned effects of mushroom treatment on this process.

With regard to FH, the expression of this enzyme was upregulated in mushroom-treated mice, showing that the citric acid cycle is not entirely inhibited; and therefore, fumarate is still produced and in need of conversion to malate. Fumarate has been shown to activate the angiogenic factor HIF1α, therefore augmenting the cancer's ability to sustain and spread through increasing its blood supply (27). Upregulation of FH expression by mushrooms would inhibit fumarate buildup in the cell and downregulate the angiogenic response. Additionally, endothelin 1, which is a gene that is commonly upregulated in hypoxia (28), was downregulated threefold in tumor specimens from mushroom-treated mice. Therefore, through analysis of the microarray data, we can further hypothesize that mushroom decreased tumor size, tumor cell proliferation, and increased tumor cell apoptosis through its effects on the TCA cycle and fatty acid metabolism. Future studies will address this hypothesis.

Several studies have demonstrated that several mushroom species can enhance immunity through activation of natural killer (NK) cells (29) and modulation of lymphocyte number and activity (30). Studies have also shown that mushroom treatment can induce both Type 1 and Type 2 immune response (31). Evasion of the immune response is one way in which tumor cells survive; therefore, the effect of mushroom on the expression of genes important to immune function, such as was seen in the microarray analysis (Table 2), is another mechanism that could contribute to its anticancer action in vivo.

Identification of the phytochemicals present in mushroom extract is important to understanding its mode of action and could facilitate future studies by enabling the use of purified compounds from the extract. Similar to our previous findings in breast cancer, the current study showed that CLA inhibited the proliferation of prostate cancer cell lines. Much research has been published illustrating the antiproliferative (25,32,33) and proapoptotic (34–36) activity of CLA. CLA has also been shown to have significant effects on the immune system such as increased T-cell responsiveness and splenocyte IL-2 production in mice fed 1% CLA in the diet (37). Thus, the presence of this compound in white button mushrooms likely significantly adds to the biological activity of the whole extract.

TABLE 1
Gene Networks Regulated by Mushroom Extract in DU-145 Derived Tumors

Molecules in Network	Score	Focus Genes	Top Functions
AKR1C2, BDKRB2, COL1A1, COL3A1, DAD1, DUSP4, EDN1, EGF, EREG, HES1, HSD17B1, IGFBP3, KIT, KITLG, MAML1, MAML2, MAML3 (includes EG:55534), MARCKS, MCL1, NF1, NOTCH3, PDCD4, PPAP2B, PTPRO, RAB5A, RPS6KB2, SCNN1A, SERPINH1, SGK, SNAI2, TPM4, VEGF, WDR1, WEE1, XRCC4	29	18	Cellular growth and proliferation, lipid metabolism, small molecule biochemistry
ADCYAP1, ADFP, ADH7, AKR1B10, ALDH3A1, CBS, CEBPA, CLIC4, CRLF1, DHRS3, DMBT1, G1P3, GSTT1, HSPA4, IDH2, LCK, LITAF, MAP3K14, MAT2A, NFE2L2, NFIX, OAS2, PGD, QKI, RPS11, S100A8, S100A9, SLC1A4, SLC2A3, SOX4, SP1, TFAM, TNF, UBC, WDR48	18	13	Inflammatory disease, gene expression, cell death
ALPL, ALPP, BHLHB2, BMP2K, BUB1, CASP3, CLIC4, COL4A1, CTSW, DAPK1, DFFA, DFFB, EPRS, HIF1A, IL2, JAG2, MASK, MBP, MFAP5, NDRG1, NOTCH1, NT5E, P4HA1, PBK, PDE4B, PLOD2, PRODH, PTK9, PXN, RNF5, TGFB1, THRAP2, TP53, TP53RK, ZFHX1B	18	13	DNA replication, recombination, and repair, cancer, tumor morphology
API5, ATG5, ATG12, BAMBI, CEACAM6, CTNNB1, ENPP2, ESR2, FADD, FAS, FGF2, HIPK3, HSPA4L, ID4, IFITM1, IL15, MYOD1, NP, NPTX1, PDE4B, PGK1, PLXNA1, PTK7, PTPN13, PTPRG, RBM17, SEMA3C, SPARC, SRC, SURB7, TCF7, THBS4, TNS1, TRFP, ZNF45	18	13	Cell Death, Cellular Growth and Proliferation, Cell Morphology
ABCC2, AP1G1, AP1S1, CD44, CD53, COPA, CPB2, CSPG6, DHX9, DUSP6, EMP2, FCGRT, FXR1, HBE1, HOXA9, HRAS, ICAM3, IL6, IQGAP1, ITGB1, KIT, LGALS1, MYB, NLK, NXF1, NXT2, RANBP9, RDX, RPL27A, SPBC25, SPN, ST6GAL1, TOP2A, TYR, WSB1	18	13	Cell death, hematological disease, immunological disease
ACLY, AP2A2, AP2B1, AP2M1, AP2S1, ATP6V0E, CDC40, EPN1, EPS15, GOSR1, GOSR2, GPAA1, HRB, INS1, ITSN1, NFYB, PICALM, PTH, RAB5A, RRM2, SCAMP1, SIRPA, SLC34A1, SLC34A2, SLC39A8, SLC6A3, SNAP23, SNAP25, STX16, SYNJ1, TFG, USP10, VAMP3, VTI1B, YKT6	13	10	Cellular assembly and organization, cellular function and maintenance, cellular movement

TABLE 2
Functional Grouping of Mushroom Extract-regulated Genes

Symbol	Entrez Gene	Fold Change	Description
Apoptosis, cell death, cytotoxicity, viability, survival, proliferation, and growth			
ATG5	9474	2.22	Autophagy related 5 homolog (S. cerevisiae)
CEACAM6	4680	–2.22	Carcinoembryonic antigen-related cell adhesion molecule 6
CLIC4	25932	2.90	Chloride intracellular channel 4
DAPK1	1612	–2.28	Death associated protein kinase
DFFA	1676	–2.10	DNA fragmentation factor alpha polypeptide
EDN1	1906	–3.00	Endothelin 1
EGF	1950	–2.21	Epidermal growth factor
EMP2	2013	–2.20	Epithelial membrane protein 2

(Continued on next page)

TABLE 2
Functional Grouping of Mushroom Extract-regulated Genes *(Continued)*

Symbol	Entrez Gene	Fold Change	Description
FAS	355	2.84	Tumor necrosis factor (TNF) receptor family member 6
HES1	3280	–2.75	Hairy and enhancer of split 1
IGFBP3	3486	–2.31	Insulin-like growth factor binding protein 3
IL15	3600	2.41	Interleukin 15
KIT	3815	–4.00	v-kit hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KITLG	4254	2.50	KIT ligand
LGALS1	956	2.64	Lectin, galactoside-binding soluble, 1
MCL1	4170	2.21	Myeloid cell leukemia sequence 1
NF1	4763	–2.10	Neurofibromin 1
RBM17	84991	2.10	RNA binding motif protein 1
S100A8	6279	3.49	S100 calcium binding protein A8
S100A9	6280	2.01	S100 calcium binding protein A9
SGK	6446	2.20	Serum/glucocorticoid regulated kinase
SPC25	57405	2.51	NDC80 kinetochore complex component
VEGF	7422	–2.03	Vascular endothelial growth factor
WIF1	11197	–5.03	WNT inhibitory factor 1
XRCC4	7518	2.12	X-ray repair complementing defective repair in Chinese hamster cells 4
Lipid metabolism			
BDKRB2	624	2.95	Bradykinin receptor B2
DHRS3	9249	4.21	Dehydrogenas/reductase (SDR family) member 3
EDN1	1906	–3.00	Endothelin 1
EGF	1950	–2.21	Epidermal growth factor
FAS	355	2.84	TNF receptor family member 6
KIT	3815	–4.00	v-kit hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KITLG	4254	2.50	KIT ligand
LGALS1	1956	2.64	Lectin, galactoside-binding soluble, 1
MARCKS	4082	2.21	Myristoylated alanine-rich protein kinase C substrate
S100A	86279	3.49	S100 calcium binding protein A8
S100A	96280	2.01	S100 calcium binding protein A9
Immune response			
ALPP	250	–4.20	Alkaline phosphatase, placental
FAS	355	2.84	TNF receptor family member 6
IL15	3600	2.41	Interleukin 15
KIT	3815	–4.00	v-kit hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KITLG	4254	2.50	KIT ligand
LGALS1	1956	2.64	Lectin, galactoside-binding soluble, 1
NF1	4763	–2.10	Neurofibromin 1
S100A8	86279	3.49	S100 calcium binding protein A8
S100A9	96280	2.01	S100 calcium binding protein A9
VEGF	7422	–2.03	Vascular endothelial growth factor

Taken together with research from other laboratories, our results support the recommendation of white button mushroom as a dietary component that may aid in the prevention of prostate cancer in men. The in vivo studies demonstrate that oral intake of the extract is effective in the inhibition of prostate tumor growth in mice. Importantly, the dosage used in the in vivo studies is considered physiologic, as the common conversion factor from murine to human dosage is 25,

calculated on body surface area (38). In addition to the inclusion of whole mushrooms into the diet, our work with the extract and isolation of CLA from the extract suggests that purified compounds or mixtures of compounds from mushroom may have efficacy as potential dietary supplements. Future study into the mechanisms of action of mushroom extract will help us to further delineate possible roles of mushroom phytochemicals in the prevention and treatment of prostate cancer.

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