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Agaricus blazei (Class Basidiomycotina) Aqueous Extract Enhances the Expression of c-Jun Protein in MCF7 Cells

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The edible mushroom *Agaricus blazei* Murill is considered a health food in many countries after it was reported to be a source of antitumor and immunoactive compounds. An aqueous extract (AE) from this basidiomycete significantly enhanced the expression of the c-Jun/activator protein-1 (AP1) in the human breast cancer cell line MCF7. Incubating the cells with 17-β-estradiol (E2), *p*-nonylphenol (NP), and the AE combined, or NP plus the AE, resulted in increased cell proliferation compared to the untreated control by 93 and 67%, respectively. However, incubating the cells with the extract alone did not enhance cell division. It is suggested that the enhanced proliferation of MCF7 cells in the presence of NP and the AE may be due to the involvement of an AP1 gene regulatory complex. This is the first report showing enhanced c-Jun/AP1 expression in MCF7 cells incubated with an aqueous fungal extract.

KEYWORDS: ***Agaricus blazei* Murill; c-Jun; AP1; MCF7; *p*-nonylphenol; 17-β-estradiol**

INTRODUCTION

The health food and edible fungus *Agaricus blazei* Murill (ABM) has recently been identified as a source of anticarcinogens, immunomodulators, antimutagens, and bactericidal substances (*1*−*4*). Studies have shown that compounds such as α-1-4-glucan, β-1-6 glucan, and polysaccharide−protein complexes that are found in fungal extracts such as ABM enhance in vivo and in vitro cell-mediated immune responses and act as biological response modifiers (*5*, *6*). ABM is available as whole, freeze-dried mushrooms or packaged as teas, capsules, or concentrated liquid extracts. Whole mushrooms are often added to soups, sauces, or hot teas.

After screening various kinds of foodstuffs containing food factors that are known to elicit molecular changes in cells, we decided to focus our attention on *A. blazei* Murill. In this study, we exposed the human breast cancer cell line MCF7 to an aqueous, hot water extract (AE) from ABM. We also exposed the cells to 17-β-estradiol (E2), a natural estrogen, and to *p*-nonylphenol (NP), a proven xenoestrogen, as well as to combinations of these compounds with or without AE to determine their proliferative effect on MCF7 cells. Using western blotting, we also examined the expression of the c-Jun protein of the AP1 gene regulatory complex in MCF7 cells to determine whether any of the observed results were related to the expression of this protein. We assayed for the possible involvement of protein kinase C (PKC), whose activation leads to the expression of the c-Jun protein (*7*), which results in increased cell proliferation in certain cells. Finally, we assessed putative c-*jun* mRNA expression in MCF7 cells subjected to the same treatments as those in the western blotting and cell proliferation assays.

The MCF7 human breast cancer cell line possesses estrogen receptors (ER) and responds in culture to the presence of estrogens by a proliferation response. In contrast to binding assays, this has the advantage of being a biological response, which can be taken as a measure of the direct interaction of agonists with the ER and equated with estrogenic potential (*8*). Cell proliferation can be quantified by a colorimetric MTT assay (*9*, *10*).

Xenoestrogens or hormonally active agents such as NP can recognize the ER binding site and trigger cell proliferation in estrogen-responsive cells (*8*, *11*, *12*). Such compounds can also antagonize the effect of natural hormones, react directly or indirectly with them, alter the natural pattern of synthesis of hormones, and even alter hormone receptor levels (*13*).

The significant role of the activator protein-1 (AP1) in transcriptional activation was shown by Paech et al. (*14*). The ligand-bound ER can bind to the classical estrogen response element (ERE) and mediate gene transcription, but it can also bind to the heterodimeric AP1 transcription factors Fos and Jun, which recognize a transcription response element on DNA called the AP1 binding site. This binding also confers a transcriptional response, which may be proliferation or differentiation.

In transactivation experiments, Paech et al. (*14*) has shown that the drug tamoxifen inhibits the transcription of genes that are regulated by the classical ERE. However, under the control of an AP1 element, tamoxifen can also activate gene transcription. This reversal in the pharmacology of the drug helps explain

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Table 1. Experimental Setups Used in the Modified E-Screen Assay Using MTT

setup	*p*-nonylphenol[a] (1 μM)[b]	17-β-estradiol[a] (1 nM)[b]	aqueous extract[a] (5% v/v)[b]
1	+	−	−
2	+	+	+
3	+	+	−
4	+	-	+
5	−	+	−
6	−	+	+
7	−	−	+
8	−	−	−

[a] +, present; −, absent. [b] Final concentration.

why prolonged tamoxifen use to control breast cancer sometimes results in cancer of the uterus (*14*).

Protein kinase C, an enzyme involved in protein phosphorylation, is widely known to be part of the transmembrane signal transduction mechanism in eukaryotic cells. The link between protein kinase C and the AP1 complex was reviewed by Karin (*7*). Briefly, activation of PKC inhibits protein kinases that phosphorylate the regulatory site of c-Jun or activates a protein phosphatase that recognizes this site. The dephosphorylation of the regulatory site increases the DNA-binding activity of preexisting c-Jun and results in increased occupancy of the AP1 site in the c-*jun* promoter, followed by induction of c-*jun* transcription (*7*).

In this study, we determined whether the aqueous *Agaricus* extract contains compounds that can cause MCF7 cells to proliferate. We also examined if it enhances or diminishes the estrogenic potential of xenoestrogens, such as NP. Furthermore, we detected the expression of the c-Jun element of the AP1 protein complex in MCF7 cells, determined if PKC is involved, and assessed putative c-*jun* mRNA levels.

MATERIALS AND METHODS

Strains and Culture Maintenance. *A. blazei* (ATCC 76739) was grown in 100 mL of YM broth (g/L: yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10) in 300 mL flasks at 25 °C using a reciprocal shaker for 2 weeks, and the mycelia were harvested for aqueous extraction. The MCF7 cell line was obtained from Dr. H. Shinmoto of the National Food Research Institute (Tsukuba, Japan) and routinely maintained in phenol-red-free RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 1% streptomycin (5000 IU mL^{-1})−penicillin (5000 μg mL^{-1}) solution (ICN Biomedicals Inc.) in 75 cm^2 tissue culture flasks. Cells were incubated at 37 °C in a 95% air−5% CO_2 incubator. Cell passage was carried out at 80% confluence at 1:3 ratio using trypsin.

Chemicals and Extracts. *p*-Nonylphenol (Kanto Chemical Co.,), 17-β-estradiol (Wako), and an aqueous extract from the fungus *A. blazei* were used singly or in combination to treat the human breast cancer cell line MCF7. The aqueous extract was obtained by boiling 22.57 g (wet weight) of mycelium in 180 mL of distilled deionized water for 10 min. After cooling, the aqueous portion was decanted and passed through a 0.22 μm filter (Millipore) for use in the experiments.

Cell Proliferation Assay. MCF7 cells were trypsinized and plated onto 96-well plates at initial concentrations of 3000 cells per well. After the cells had been allowed to attach for 24 h, the seeding medium (10% FBS in phenol-red-free RPMI) was removed and replaced with the experimental medium (10% charcoal-treated FBS in phenol-red-free RPMI). *p*-Nonylphenol, 17-β-estradiol, and the aqueous extract were then added according to **Table 1**. A separate experiment was done to evaluate the effects of various concentrations of nonylphenol on the survival and proliferation of MCF7 cells. The plates were incubated for 6 days, after which time 10 μL of 50 μg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Dojindo) was added to each well and then incubated for 4 h. Sodium dodecyl sulfate (10%) was then added at 100 μL per well, and the plates were incubated for 24 h. The absorbance was then measured at 570 nm using a microplate reader.

Table 2. Experimental Setups Used in the Protein Kinase C Assay

setup	*p*-nonylphenol[a] (1 μM)[b]	17-β-estradiol[a] (1 nM)[b]	aqueous extract[a] (5% v/v)[b]
1	−	+	−
2	+	−	−
3	−	−	−
4	−	+	+
5	+	−	+
6	−	−	+

[a] +, present; −, absent. [b] Final concentration.

Western Blotting. The experimental setups shown in **Table 1** were also used in the immunoblotting experiments. Petri plates were inoculated with MCF7 cells at 1×10^5 cells/plate in 10 mL of medium and allowed to attach for 24 h. The seeding medium was then removed and replaced with 10 mL of experimental medium, and the test compounds were added. After the plates had been incubated for 6 days, the medium was removed and the cells were washed with PBS(−) (Nissui). After complete removal of the PBS(−), 1 mL of RIPA buffer (PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 0.6 mM PMSF, 30 μL/mL aprotinin, and 1 mM sodium orthovanadate) was added to lyse the cells. The protein concentrations in each of the samples were then determined using the DC protein assay (Bio-Rad). Equal amounts of protein were then precipitated using methanol and chloroform, followed by drying at room temperature and addition of 100 μL of sample buffer [mL/10 mL solution: 0.5 M Tris-HCl (pH 6.8), 1; 10% SDS, 2; β-mercaptoethanol, 0.6; glycerol, 1; distilled deionized water, 5.4; plus a few drops of 1% bromophenol blue]. The samples were then placed in a heat block for 5 min at 95 °C to completely denature the proteins. This was followed by SDS-PAGE using a 10% acrylamide gel. After electrophoresis, the gel was rinsed twice in transfer buffer (39 mM glycine, 48 mM Tris, and 20% methanol), and the proteins were transferred to Immuno-Blot PVDF membranes (Bio-Rad) using a semidry transfer cell (Bio-Rad). The membrane was then rinsed twice, 15 min each time, with TBST (6.06 g of Tris, 8.76 g of NaCl, 0.5 mL of Tween 20 in 1 L of distilled deionized water) followed by incubation with 1× Block Ace (Dainihon Pharmaceutical Industry) to prevent nonspecific binding. After washing, the membrane was incubated for 90 min with 500× diluted c-Jun/AP1 polyclonal rabbit IgG (Calbiochem), washed, and then incubated with a 2000× diluted goat anti-rabbit IgG horseradish peroxidase-conjugated affinity-purified antibody (Chemicon) for 1 h. The membrane was then washed five times with TBST and then incubated for 5 min with ECL plus western blotting detection reagents (Amersham Pharmacia Biotech), and the bands were revealed using a Kodak BioMax MR film after 24 h of exposure.

Protein Kinase C (PKC) Assay. Table 2 shows the experimental setups used in the PKC assay. About 1×10^6 cells were plated onto sterile Petri plates and cells allowed to attach for 24 h. After 24 h, the seeding medium was removed and replaced with the experimental medium.

The test compounds were then added and the plates incubated for 24, 72, and 144 h. Setups that were incubated for 144 h started with 1×10^5 cells per plate. The cells were then washed twice with equal volumes of PBS(−) and then incubated with lysis buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 50 mM sodium fluoride, 5 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM sodium vanadate, 1% (v/v) Nonidet P-40 plus 1 mM PMSF, 5 μg/mL leupeptin hydrochloride, 1 μM E64, and 50 mM mercaptoethanol] for 10 min at 4 °C. The cells were then collected using a sterile plastic cell scraper, transferred to sterile centrifuge tubes, and spun at 15300 rpm for 10 min at 4 °C. The supernatant was then used for the PKC assay using the Pep Tag assay kit (Promega).

RNA Isolation and Northern Blot Hybridization. The experimental setups shown in **Table 1** were also used in the northern blotting

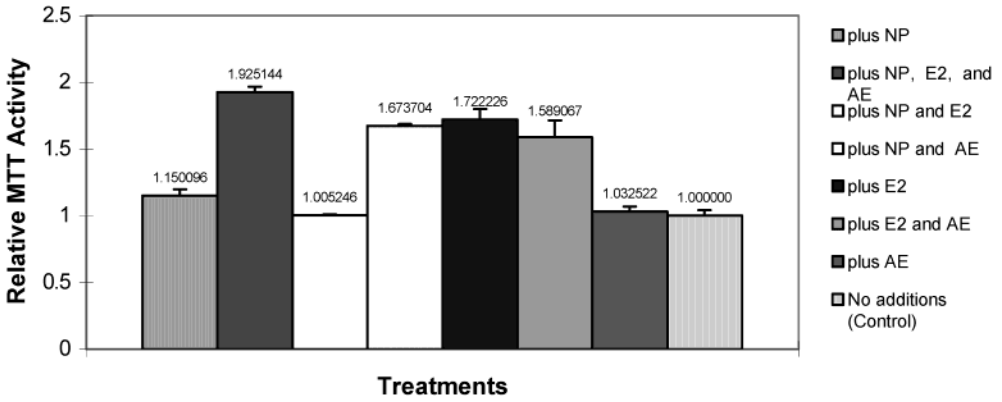

Figure 1. MTT activity of MCF7 cells in the presence or absence of 1 μM *p*-nonylphenol (NP), 1 nM 17-β-estradiol (E2), and 5% (v/v) *Agaricus* extract (AE). Results based on at least four independent replicates are expressed as relative MTT activity, which is obtained by dividing the mean MTT activity of the cells incubated with the test compounds and/or AE by the mean activity of the control (no additions), ± SD. Relative MTT activity of control is therefore 1.

experiments. Petri plates were inoculated with MCF7 cells at 1×10^5 cells/plate in 10 mL of medium and allowed to attach for 24 h. The seeding medium was then removed and replaced with 10 mL of experimental medium, and the test compounds were added. After the plates had been incubated for 6 days, the medium was removed and the cells were washed three times with 1 mL of ice-cold PBS(−) (Nissui). One milliliter of ice-cold PBS(−) was then finally added, and the cells were scraped and transferred to centrifuge tubes on ice. The cells were then centrifuged for 5 min at 4 °C and 1000 rpm, the supernatant was discarded, and total RNA was then isolated from the cells using the Isogen-LS (Nippon Gene) protocol. The RNA pellet obtained from the confluent Petri plates was then dried and resuspended in 50 μL of DEPC-treated water. The integrity of the RNA was then assessed via agarose gel electrophoresis.

Ten microliters of the RNA suspension for each sample was electrophoresed in 1.2% (w/v) agarose gel containing formaldehyde (2.2 M) and 10× MOPS buffer, followed by the assessment of RNA integrity by visualizing the 18s and 28s RNA bands and the absence of streaking under UV. The RNA was then transferred by capillary blotting to a Hybond-N+ membrane (Amersham Pharmacia Biotech) overnight and UV cross-linked using a Bio-Rad GS gene linker. Blots were prehybridized, hybridized, and washed following the ECL direct nucleic acid labeling and detection systems protocol (Amersham Pharmacia Biotech). The *c-jun* probe, a 43-mer, single-stranded, antisense, synthetic DNA probe (Geneka Biotechnology), was labeled according to ECL instructions. Probe hybridization was conducted overnight at 42 °C, and the membrane was washed under low- or medium-stringency conditions and exposed to photographic film (Kodak BioMax MR) overnight.

RESULTS

Cell Proliferation Assay. Results in **Figure 1** show that when the MCF7 cells were incubated for 6 days in the presence of AE in combination with both NP and E2, there was a significant increase (93%) in cell proliferation compared to control ($P < 0.01$, *t* test), which had no additions (**Table 3**). However, cells incubated with AE alone or with E2 plus NP did not show any significant increase compared with control. Interestingly, the proliferative effect of AE, E2, and NP acting in consortium is higher than that of E2 alone (93 vs 72%) (**Table 3**). Results in **Figure 2** show the same trend, regardless of the NP concentration.

Western Blotting. Protein samples from cells incubated under different conditions as shown in **Table 1** were assayed for the expression of the c-Jun protein. Results in **Figure 3** show heightened expression in MCF7 cells incubated with AE alone (lane 7), as well as a slight but noticeable expression in cells incubated with both E2 and AE (lane 6). Apparently, there was no detection of c-Jun in lanes 1−5 and 8 (plus NP, plus NP, E2, and AE, plus NP and E2, plus NP and AE, plus E2, and untreated control, respectively).

Table 3. Percent Increase in MTT Activity of MCF7 Cells Compared to Control after Treatment with 1 μM *p*-Nonylphenol (NP), 1 nM 17-β-Estradiol (E2), and 5% (v/v) *Agaricus* Extract (AE)

cells plus	% increase in MTT activity compared to control
NP/E2/AE	93
NP/AE	67
E2	72
E2/AE	59
NP	not statistically significant compared to control[a]
NP/E2	not statistically significant compared to control[a]
AE	not statistically significant compared to control[a]

[a] *t* test; E2 vs E2/AE (significant, $P < 0.001$); E2 vs NP/E2/AE (significant, $P < 0.05$); E2 vs NP/AE (not significant, $P > 0.1$).

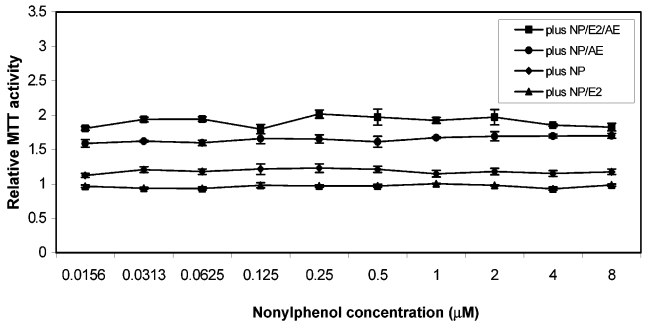

Figure 2. MTT activity of MCF7 cells in the presence of various concentrations of *p*-nonylphenol (NP), plus or minus 1 nM 17-β-estradiol (E2) and 5% (v/v) aqueous *Agaricus* extract (AE). Results based on four independent replicates are expressed as relative MTT activity, which is obtained by dividing the mean MTT activity of cells incubated with the test compounds and/or AE by the mean MTT activity of the control (no additions), ± SD. Relative MTT activity of control is therefore 1. Two-way ANOVA showed significant differences between the <plus NP/E2/AE> and <plus NP/AE> treatments compared to control ($P < 0.0001$), whereas the differences in the MTT activity of cells incubated in different NP concentrations within the same treatments are not statistically significant ($P > 0.05$).

Protein Kinase C Assay. The Pep Tag assay used in this study utilizes a brightly colored, fluorescent peptide substrate that is highly specific for PKC. Phosphorylation of this peptide by PKC alters its net charge from +1 to −1. Hence, the phosphorylated species migrates toward the anode while the

Figure 3. Western blotting results showing the expression of the c-Jun/API protein in MCF7 cells after 6 days of incubation in the presence or absence of 1 μM *p*-nonylphenol (NP), 1 nM 17-β-estradiol (E2), and 5% (v/v) aqueous *Agaricus* extract (AE): (lane 1) plus NP only; (lane 2) plus NP, E2, and AE; (lane 3) plus NP and E2; (lane 4) plus NP and AE; (lane 5) plus E2 only; (lane 6) plus E2 and AE; (lane 7) plus AE only; (lane 8) no addition (control). Results show enhanced expression of the c-Jun protein in the presence of 5% (v/v) aqueous *Agaricus* extract (lane 7).

nonphosphorylated substrate migrates toward the cathode when run on an agarose gel. Results revealed PKC activity in all experimental setups, including control (data not shown). All substrates showed migration toward the anode after incubation with protein lysates obtained from the treated cells.

Northern Blotting. Hybridization signals were not detected in total RNA samples obtained from MCF7 cells incubated with E2, NP, and AE in the same setups shown in **Table 1**. Reasons for this are discussed below.

DISCUSSION

Members of the *fos* and *jun* gene family, including the protooncogene c-*jun*, are collectively referred to as immediate early response (IER) genes. Although their role in cell proliferation requires further investigation (*7*), they are rapidly induced by mitogenic agents such as nerve growth factor (*15*), epidermal growth factor (*16*), and serum (*17*).

In this study, we observed a significant expression of the c-Jun protein in MCF7 cells after 6 days of incubation with AE (**Figure 3**). Although this is not proven conclusively, this result may be correlated with enhanced cell proliferation in MCF7 cells incubated with the estrogenic compound nonylphenol in the presence of AE.

The cell proliferation assay used in this study uses MTT to provide an estimate of the number of cells as the end point (*9*). MTT is a yellow water-soluble tetrazolium dye that is reduced by live, but not dead, cells to a purple formazan product that is insoluble in aqueous solutions (*18*). A microtitration assay such as MTT provides a simpler way of determining the extent of MCF7 cell proliferation in the presence or absence of estrogenic compounds. The concentration used for NP (1 μM) was based on previous studies (*8*), which showed it to be a full ER agonist at this concentration.

Results in **Figure 1** showing no significant difference in cell proliferation between cells incubated with AE and the control are consistent with previous studies that showed the presence of antitumor compounds from ABM (*1*). In vivo studies using tumor-bearing mice have also confirmed this finding (*19, 20*). On the other hand, the slight but not significant difference in proliferative activity of cells incubated with NP versus the control (**Figure 1**; **Table 3**) is comparable to that obtained by Villalobos et al. (*21*) using the MCF7 ATCC strain. The strain used in this study was originally obtained from ATCC. Moreover, the not significant difference in proliferative activity of cells incubated with NP plus E2 (**Figure 1**; **Table 3**) versus the control confirms previous findings that many weak estrogens have both partial estrogenic and antiestrogenic behaviors (*22*). Weak estrogens, such as NP, can compete with 17-β-estradiol for estrogen receptors but do not elicit the same pleiotropic response.

PKC assay results showed that PKC inhibition is not the reason why no c-Jun proteins were apparently detected through western blotting in some treatments (**Figure 3**), because all relevant treatments revealed PKC activity. As mentioned earlier, PKC has been conclusively shown to mediate the expression of c-*jun* mRNA in certain cells (*7, 23*).

The northern blot hybridization experiment was conducted to determine if putative levels of c-*jun* mRNA in MCF7 cells exposed to E2, NP, and AE can explain **Figure 3**. Results showed no detectable c-*jun* mRNA signal from any of the treatments and control (data not shown), despite the experiment being performed at least four times, each time with increasing care and attention to detail. This is consistent with published reports so far. Philips et al. (*24*) have shown that MCF7 cells incubated for 45 min with or without 1×10^{-7} M 17-β-estradiol did not yield any detectable c-*jun* mRNA. The c-*jun* mRNA was strongly expressed only in the presence of epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) (*24*). By transiently transfecting MCF7 cells with an AP1 inducible reporter construct, Philips et al. (*24*) have shown that ER ligands modulate AP1-induced responses by affecting c-*fos* and c-*jun* transcriptional activity but not their synthesis.

Studies using BALB/c 3T3 cells have also shown that the c-*jun* mRNA was expressed only in the presence of platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) and the expression was detectable only within 3 h of exposure to both compounds, beyond which no c-*jun* mRNA was detected (*17*). On the other hand, human oral keratinocytes exposed to 5 mM acetaldehyde expressed c-*jun* mRNA within 1 h of treatment but returned to levels similar to those in untreated cells within 6 h (*25*). These findings prove the lability of c-*jun* mRNA, which is typical of immediate early response genes such as c-*jun* and c-*fos*. We do not discount, however, the transcriptional activity and synthesis of c-*jun* mRNA despite the absence of hybridization signals in our experiment. Gygi et al. (*26*) have shown that the correlation of mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data. In some genes, although the mRNA levels were of the same value, the protein levels varied by >20-fold (*26*).

In this paper, we attempt to speculate on the results of the cell proliferation assay in view of the enhanced expression of c-Jun/AP1 in cells incubated with AE. Because AE clearly induced c-Jun protein expression and cells incubated with NP plus AE showed enhanced proliferation, we suggest that the increased proliferation of MCF7 cells incubated with NP in the presence of AE was due to the AP1 pathway. E2, on the other hand, promotes proliferation through the classical ERE. Enhanced proliferation of cells incubated with NP and E2 plus AE may have occurred using both pathways. c-Jun was not detected in any setups containing AE because if the AP1 gene regulatory complex was truly involved in proliferation, then the c-Fos/c-Jun heterodimers may have formed. The formation of this stable heterodimer will prevent anti-Jun antibodies from recognizing the c-Jun protein in western blotting experiments (*7*).

In conclusion, this study has shown that an aqueous extract from the edible fungus *A. blazei* Murill induced c-Jun protein expression in MCF7 cells and that the enhanced proliferation of MCF7 cells in the presence of nonylphenol plus the aqueous extract may be due to the involvement of the AP1 gene regulatory complex.

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