CHAPTER 4

AGARICUS BLAZEI (CLASS BASIDIOMYCOTINA) AQUEOUS EXTRACT ENHANCES THE EXPRESSION OF C-JUN PROTEIN IN MCF7 CELLS

4.1. 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 (Takaku et al., 2001; Fujimiya et al., 1998; Fujimiya et al., 1999; Osaki et al., 1994). 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 (Ooi and Liu, 2000; Itoh et al., 1994). 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 examining various kinds of foodstuffs containing food factors that are known to elicit molecular changes in cells, the author decided to focus his attention on *A. blazei* Murill. In this study, he exposed the human breast cancer cell line MCF7 to an aqueous, hot water extract (AE) from ABM. He 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, he 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. He assayed for the possible involvement of protein kinase C (PKC), whose activation leads to the expression of the c-Jun protein (Karin, 1991), which results in increased cell proliferation in certain cells. Finally, he 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 (Soto et al., 1995). Cell proliferation can be quantified by a colorimetric MTT assay (Mosmann, 1983; Mäkelä et al., 1995).

Xenoestrogens or hormonally active agents such as NP can recognize the ER binding site and trigger cell proliferation in estrogen-responsive cells (Soto et al., 1991; White et al., 1994; Soto et al., 1995). 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 (Committee on Hormonally Active Agents in the Environment, 1999).

The significant role of the activator protein-1 (AP1) in transcriptional activation was shown by Paech et al (1997). 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. (1997) 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 why prolonged tamoxifen use to control breast cancer sometimes results to cancer of the uterus (Paech et al., 1997).

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 (1991). 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 pre-existing c-Jun and results in increased occupancy of the AP1 site in the c-*jun* promoter, followed by induction of c-*jun* transcription (Karin, 1991).

In this study, the author determined whether the aqueous *Agaricus* extract contains compounds (food factors) that can cause MCF7 cells to proliferate. He also examined if it enhances or diminishes the estrogenic potential of xenoestrogens, such as NP. Furthermore, he 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.

4.2. Materials and Methods

Strains and culture maintenance

Agaricus blazei (ATCC 76739) was grown in 100 ml 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 two weeks and the mycelia harvested for aqueous extraction. The MCF 7 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 I.U. ml^{-1}) – penicillin (5000 μg ml^{-1}) solution (ICN Biomedicals Inc.) in 75-cm 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 Company, Japan), 17 β-estradiol (Wako, Japan), and an aqueous extract from the fungus Agaricus blazei were used singly or in combination to treat the human breast cancer cell line MCF-7. The aqueous extract was obtained by boiling 22.57 g (wet weight) of mycelium in 180 ml distilled deionized water for 10 minutes. After cooling, the aqueous portion was decanted and passed through a 0.22 μm filter (Millipore) for use in the experiments.

Cell Proliferation Assay

MCF-7 cells were trypsinized and plated onto 96-well plates at initial concentrations of 3,000 cells per well. After allowing the cells 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 varying concentrations of nonylphenol on the survival and proliferation of MCF-7 cells. The plates were incubated for 6 days, after which 10 µl of 50 µg/ml MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Dojindo, Japan) 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 incubated for 24 h. The absorbance was then measured at 570 nm using a microplate reader.

Table 1. Experimental set-ups used in the modified E-screen assay using MTT

Set-up	*p*-Nonylphenol (1 µM)*	17 β-estradiol (1 nM)*	Aqueous extract (5% v/v)*
1	+	-	-
2	+	+	+
3	+	+	-
4	+	-	+
5	-	+	-
6	-	+	+
7	-	-	+
8	-	-	-

+, present; -, absent * Final concentration

Western Blotting

The experimental set-ups shown in **Table 1** were also used in the immunoblotting experiments. Petri plates were inoculated with MCF-7 cells at 1 x 10^5 cells / plate in 10 ml medium and allowed to attach for 24 h. The seeding medium was then removed, replaced with 10 ml experimental medium, and the test

compounds added. After incubating the plates for 6 days, the medium was removed and the cells washed with PBS(-) (Nissui, Japan). After completely removing the PBS (-), 1 ml RIPA buffer (PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 0.6 mM PMSF, 30 μl/ml Aprotinin, 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 (Biorad). Equal amounts of protein were then precipitated using methanol and chloroform, followed by drying in room temperature, and addition of 100 μl 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, 20% methanol) and the proteins transferred to Immuno-Blot™ PVDF membranes (Biorad) using a semi-dry transfer cell (Biorad). The membrane was then rinsed twice, 15 min each time, with TBST (6.06 g Tris, 8.76 g NaCl, 0.5 ml Tween 20 in 1 li distilled deionized water) followed by incubation with 1X Block Ace (Dainihon Pharmaceutical Industry, Japan) to prevent non-specific binding. After washing, the membrane was incubated for 90 min with 500X diluted c-Jun/AP1 polyclonal rabbit IgG (Calbiochem), washed, and then incubated with a 2000X diluted goat anti-rabbit IgG horseradish peroxidase-conjugated affinity purified antibody (Chemicon, U.S.A.) for 1 h. The membrane was then washed 5 times with TBST, then incubated for 5 min with ECL Plus Western blotting detection reagents (Amersham

Pharmacia Biotech) and the bands revealed using a Kodak BioMax MR film after 24 h exposure.

Protein Kinase C Assay

Table 2 shows the experimental set-ups used in the PKC assay. About 1 x 10^6 cells were plated onto sterile Petri plates and cells allowed to attach for 24 h. After which, the seeding medium was removed and replaced with the experimental medium.

Table 2. Experimental set-ups used in the protein kinase C assay

Set-up	*p*-Nonylphenol (1 μM)*	17 β-estradiol (1 nM)*	Aqueous extract (5% v/v)*
1	-	+	-
2	+	-	-
3	-	-	-
4	-	+	+
5	+	-	+
6	-	-	+

+, present; -, absent * Final concentration

The test compounds were then added and the plates incubated for 24, 72 and 144 h. Set-ups that were incubated for 144 h started with 1 x 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 1mM 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 set-ups shown in Table 1 were also used in the northern blotting experiments. Petri plates were inoculated with MCF-7 cells at 1 x 10^5 cells / plate in 10 ml medium and allowed to attach for 24 h. The seeding medium was then removed, replaced with 10 ml experimental medium, and the test compounds added. After incubating the plates for 6 days, the medium was removed and the cells washed thrice with 1 ml ice-cold PBS(-) (Nissui, Japan). One ml of ice-cold PBS (-) was then finally added and the cells scraped and transferred to centrifuge tubes on ice. The cells were then centrifuged for 5 min, 4 °C at 1000 rpm, the supernatant discarded, and total RNA was then isolated from the cells using the Isogen-LS (Nippon Gene, Japan) 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 µl of the RNA suspension for each sample were electrophoresed in 1.2% (w/v) agarose gel containing formaldehyde (2.2 M) and 10X 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 Hybond-N+ membrane (Amersham Pharmacia Biotech, USA) overnight and UV cross-linked using a Biorad GS Gene Linker. Blots were prehybridized, hybridized, and washed following the ECL direct nucleic acid labelling and detection systems protocol (Amersham Pharmacia Biotech, USA). The c-jun probe, a 43-mer,

single-stranded, antisense, synthetic DNA probe, was labelled according to ECL instructions. Probe hybridization was conducted overnight at 42 °C, the membrane washed under low or medium-stringency conditions and exposed to photographic film (Kodak BioMax MR) overnight.

4.3. Results

Cell Proliferation Assay

Results in **Figure 8** *(page 76)* 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 that of 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 9** *(page 77)* show the same trend, regardless of the NP concentration.

Table 3. Percent increase in MTT activity of MCF7 cells compared to control after treatment with 1 μM nonylphenol (NP), 1 nM 17 β-estradiol (E2) and 5% (v/v) Agaricus extract (AE).

Cells plus	Percent 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	
AE	

[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, $P>0.1$)

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 10** *(page 78)* 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 to 5 and 8 (plus NP, plus NP, E2 & AE, plus NP & E2, plus NP & AE, plus E2, and untreated control, respectively).

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 non-phosphorylated substrate migrates toward the cathode when run on an agarose gel. Results revealed PKC activity in all experimental set-ups, including control (**Figure 11**, *page 79*). All substrates showed migration towards 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 set-ups shown in Table 1. Reasons for this are discussed below.

4.4. Discussion

Members of the *fos* and *jun* gene family, including the protooncogene c-*jun*, are collectively referred to as immediate early response (IER) genes. While their role in cell proliferation requires further investigation (Karin, 1991), they are rapidly induced by mitogenic agents such as nerve growth factor (Wu et al., 1989), epidermal growth factor (Quantin and Breathnach, 1989) and serum (Ryder and Nathans, 1998).

In this study, the author observed a significant expression of the c-Jun protein in MCF7 cells after 6 days incubation with AE (**Figure 10**). While 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 (Mosmann, 1983). 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 (Vistica et al., 1991). A microtitration assay such as MTT provides a simpler way of determining 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 (Soto et al., 1995), which showed it to be a full ER agonist at this concentration.

Results in **Figure 8** 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 (Takaku et al., 2001). *In vivo* studies using tumor-bearing mice have also confirmed this finding (Fujimiya et al., 1998; Ito et al., 1997). On the other hand, the slight but not significant

difference in proliferative activity of cells incubated with NP versus the control (**Figure 8, Table 3**) is comparable to that obtained by Villalobos et al. (1995) 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 8, Table 3**) versus the control confirms previous findings that many weak estrogens have both partial estrogenic and antiestrogenic behaviors (Tham et al., 1998). 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 (**Figure 11**) showed that PKC inhibition is not the reason why no c-Jun proteins were apparently detected through western blotting in some treatments (**Figure 10**), since 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 (Karin, 1991; Chen et al., 2001).

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 10**. 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 4 times, each time with increasing care and attention to detail. This is consistent with published reports so far. Philips et al. (1993) 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) (Philips et al., 1993). By transiently transfecting MCF7 cells with an AP1 inducible reporter construct, Philips et al. (1993) 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 hours of exposure to both compounds, beyond which no c-jun mRNA was detected (Ryder and Nathans, 1998). On the other hand, human oral keratinocytes exposed to 5 mM acetaldehyde expressed c-*jun* mRNA within one hour of treatment but returned to levels similar to those in untreated cells within 6 hours (Timmons et al., 2002). These findings prove the lability of c-*jun* mRNA, which is typical of immediate early response genes such as c-*jun* and c-*fos*. The author does not discount, however, the transcriptional activity and synthesis of c-*jun* mRNA despite the absence of hybridization signals in this experiment. Gygi et al. (1999) have shown that the correlation of mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data. In some genes, while the mRNA levels were of the same value the protein levels varied by more than 20-fold (Gygi et al., 1999).

In this work, the author attempts 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 that cells incubated with NP plus AE showed enhanced proliferation, the author suggests 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, E2 plus AE may have occurred using both pathways. c-Jun was not detected in all set-

ups 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 (Karin, 1991).

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.

4.5. Summary

The edible mushroom *Agaricus blazei* Murill is considered a health food in many countries after it was reported as 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 AP1 gene regulatory complex. This is the first report showing enhanced c-Jun/AP1 expression in MCF7 cells incubated with an aqueous fungal extract.