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Brefeldin A Is an Estrogenic, Erk1/2-Activating Component in the Extract of *Agaricus blazei* Mycelia

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Supporting Information

ABSTRACT: We purified an Erk1/2-activating component in *Agaricus blazei* and identified it as brefeldin A (BFA). The extract of *A. blazei* mycelia (ABE) previously showed an estrogenic gene-expression profile and positive effects in patients with cardiovascular symptoms. Here, we demonstrate that BFA has estrogenic activity in reporter gene assays and stimulates an estrogen-receptor pathway revealed by activation of Erk1/2, although BFA had no growth-stimulating activity in breast cancer MCF-7 cells. The presence of estrogenic activity without any explicit growth-stimulating effect is unique to BFA, and such components are termed here "silent estrogens". To test this hypothesis, we examined the target-gene transcription and signaling pathways induced by BFA. Furthermore, BFA was found in the mycelium but not fruiting body of *A. blazei*, suggesting the potential use of ABE for therapeutics and its supplementary use in traditional medicines and functional foods.

KEYWORDS: *brefeldin A, cardiovascular disease, cell growth, estrogen, Erk1/2, signal transduction*

INTRODUCTION

Agaricus blazei Murrill (*A. blazei*) is a medicinal mushroom, commonly known as Himematsutake in Japan. Its extracts have been tested experimentally used against leukemia[1] and gynecological cancer,[2] because they contain β-glucans, compounds known to stimulate the immune system[3,4] or activate NK cells,[2] and other components that inhibit tumor growth or metastasis by inducing apoptosis[5,6] and/or inhibiting angiogenesis.[7,8] The mechanism by which the immune system is stimulated is not well understood, although *A. blazei* was reported to facilitate the production of cytokines, such as interferons and interleukins.[9] *A. blazei* is used by some 500000 people in Japan[10] in complementary and alternative medicine[11] and/or as a supplement. *A. blazei* is also known to have antiviral, antibacterial, and antifungal activities in cell culture,[9] although its ability to inhibit viruses in the human body has not been studied. Other reported effects include a lowering of blood cholesterol/glucose levels,[12,13] inhibition of pathogenic factors,[14−16] and improvement of insulin resistance.[17] However, there is little clinical evidence to support these findings.

Previously, we reported estrogen-like activity in an extract of *A. blazei* mycelia (ABE),[18] which was supported by preliminary clinical observations including an increase in oxidized low-density lipoprotein (OxLDL) levels in hyperlipidemic patients. Improvement of experimental atherosclerotic lesions in rabbits fed a high-cholesterol diet was observed after the uptake of ABE, suggesting a mechanism similar to estrogenic action on atherosclerosis, that is, positive effects of estrogen on atherosclerosis shown by a lower incidence of atherosclerosis in premenopausal women, but not postmenopausal women, and men with cirrhosis-related hyperestrogenemia.[19,20] There is a pathway for gender-specific cardioprotection mediated by estrogen-receptor- (ER-) dependent gene regulation.[21] One interesting observation was that the extract did not show growth-stimulating activity in MCF-7 breast cancer cells, a cell line widely used for examining ER-dependent estrogenic function and expressing ERs α and β (see ref 22, for example). Thus, the above mechanism would not be exactly the same as that for the ER-dependent function, and more importantly, the lack of growth activity in tumor cells could provide insight into a new signaling pathway involved in estrogen's actions. Its potential use in a variety of estrogen treatments without stimulating cancer cells is worth noting.

Estrogen is a lipophilic steroid hormone, and therefore, it can pass through the cell membrane and interact with nuclear receptors. Thus, there are two types of ERs, designating two major estrogen-signaling pathways, genomic and nongenomic.[23,24] In the genomic pathway, the binding of estrogen to the nuclear receptor follows dimerization of the receptor and

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subsequent binding of the receptor dimer to specific sequences of DNA known as the estrogen-responsive element. The DNA/receptor complex then recruits other proteins, including coactivators such as SRC-1 and p300,[25] which are responsible for the transcription of target genes, the genes whose product proteins modulate cell function.

Conversely, the binding of estrogen to membrane receptors rapidly activates the nongenomic pathway.[26−28] In this pathway, signaling occurs very rapidly compared with gene transcription and protein synthesis, within seconds to minutes. This signaling is mediated by second messengers including nitric oxide, receptor tyrosine kinases, G-protein-coupled receptors (GPCRs), and protein kinases including phosphatidylinosiol-3-kinase (PI3K) and Akt, mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase-1/2 (Erk1/2). Cell-growth and antiapoptotic signaling of estrogen in this pathway were reported to be mediated by Erk1/2 and/or Akt after the activation of GPCRs.[29] Meanwhile, MAPK/Erk1/2 can directly phosphorylate the ER at serine 118 and up- or down-regulate the transcription of target genes.[30−32] p90 ribosomal-S6-kinase (RSK), a downstream target of MAPK, can also phosphorylate the ER at serine 167, increasing its transcriptional efficiency.[33] In breast cancer cells, estrogen activates the HER-2/PKC-delta/Ras/Erk1/2 pathway, leading to cell proliferation.[34] Activated ERs activate nitric oxide synthase (eNOS) through PI3K and Akt, which promotes the release of NO and leads to vasodilation in the vasculature.[35] Thus, signal mediators such as Erk1/2 have been used widely to monitor estrogen signaling.[36,37]

In this report, we examined whether estrogenic activity of ABE is attributable to a single component or a combination of several compounds and how the clinical and pharmacological data can be explained at the molecular level.

MATERIALS AND METHODS

Materials. Estrogen (17β-estradiol or E_2) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). *A. blazei* powder was prepared from mycelia of *A. blazei*, strain my26, by the JMCU Center Corporation. The aqueous extract of *A. blazei* mycelia (ABE) was prepared by boiling 25 g of *A. blazei* powder in 500 mL of Milli-Q water for 10 min. The supernatant was recovered by centrifugation at 3300*g* for 25 min and sterilized by filtration through 0.22-μm filters (Millipore, Billerica, MA). To prepare the ethyl acetate fraction, 200 mL of ABE was mixed with 200 mL of ethyl acetate for 10 min and centrifuged at 3300*g* for 25 min. The ethyl acetate fraction was evaporated dry, and the dried material dissolved in 80% methanol was centrifuged at 3300*g* for 10 min at 25 °C to remove debris (final concentration, 0.01 g/mL).

High-Performance Liquid Chromatography (HPLC). ABE was passed through a cartridge filter (Millex LCR, 0.2-μm pore size, Millipore) and aliquots (5 or 100 mg) were subjected to HPLC using a reverse-phase 5C-18 preparative column (4.6 × 200 mm, 10 mm ×250 mm, YMC, Kyoto, Japan) equilibrated with Milli-Q water for analytical as well as preparative purposes. The column was washed with water and eluted with a 0−100% gradient of acetonitrile in Milli-Q water. The eluate was monitored by measuring optical absorbance at 210 nm (OD_{210}), and the fractions collected were concentrated by evaporation and then subjected to Western blotting to identify the fractions showing higher Erk1/2 activity.

Western Blotting. MCF-7 cells were obtained from the JCRB Cell Bank (National Institute of Health Sciences, Tokyo, Japan) and cultured in RPMI1640 (Invitrogen, Carsbad, CA) medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells harvested at 90% confluence by the addition of 2 mL of trypsin were plated in phenol-red-free RPMI1640 medium in 6-cm plates at a density of 10^5 cells per well. The cells were serum-starved in phenol-red-free RPMI 1640 medium for 48 h and then treated with ABE (10 μg/mL), 10 nM E_2, or vehicle (0.1% v/v DMSO). For the assay with various signal inhibitors, cells were first cultured in phenol-red-free RPMI1640 medium supplemented with 10% dextran-coated charcoal-treated FBS (DCC-FBS) on 10-cm plates for 2 days and then transferred into serum-free medium and cultured for one more day. The cells were then pretreated with 1 μM ICI 182,780 (Tocris Bioscience, Bristol, U.K.) for 1 h, 50 μM LY 294002 (Calbiochem/Merck KGaA, Darmstadt, Germany) or 10 nM rapamycin (Sigma-Aldrich) for 30 min, which was followed by the treatment with 10 nM E_2, 10 nM BFA, or vehicle (0.1% v/v DMSO). Total protein was extracted by mixing with an equal volume of sodium dodecyl sulfate (SDS) buffer [0.125 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 10% 2-mercaptoethanol, 4% SDS, and 10% sucrose as 2× buffer] from the cells after they were sonicated on ice for 30 s. After incubation at 95 °C for 5 min with a loading buffer, protein (20 μg) was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 5−20% gradient gel and electrotransferred onto nitrocellulose membranes (Millipore, Billerica, MA) using a semidry transfer cell (BIO-RAD, Hercules, CA) at 1 mA/cm^2 for 1 h. The membranes were then blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% bovine serum albumin (TBST-BSA). The membranes were incubated with the indicated polyclonal antibodies (Cell Signaling Technologies, Ipswich, MA) overnight at 4 °C after appropriate dilution (1:1000) in TBST-BSA. The antibody/antigen complexes were detected with horseradish peroxidase-coupled goat antibodies against rabbit IgG (Cell Signaling Technologies) after dilution (1:3000) with TBST-BSA and visualized using the ECL-plus Western Blotting Detection System (Amersham Pharmacia Biotech, Arlington Heights, IL).

Structural Analysis. Structural analysis of the purified Erk1/2-activating component was performed by mass spectrometry (MS), ^{1}H and ^{13}C NMR spectrometry, optical rotation analysis, elemental composition analysis, and infrared spectrometry, and data were compared with those reported by Suh et al.[38] MS was performed by liquid chromatography/MS/MS using a quadrupole ion-trap-time-of-flight mass spectrometer, LC-20A/MS-IT-TOF (Shimadzu Corporation, Tokyo, Japan), and NMR spectrometry was performed at Tokyo Women's Medical University using an INOVA AS400 instrument (Agilent Technologies, Santa Clara, CA). The MS analysis was carried out directly by MS/MS in positive-ion mode, and the values of $[M + H]^+$ and $[M + Na]^+$ were obtained. ^{1}H NMR spectrometry was performed at 600 MHz using CD_3OD as a standard, and ^{13}C NMR spectrometry was performed at 100 MHz using CD_3OD as a standard. Optical rotation analysis was carried out by Junsei Chemical (Tokyo, Japan), and elemental composition analysis and infrared spectrometry were carried out by Toray Research Center using varioEL IRMS (Elementar, Hanau, Germany) and FTS-135 (Bio-Rad Laboratories, Benicia, CA) instruments, respectively.

Sulforhodamine B (SRB) Assay. The SRB assay was performed according to the method of Skehan et al.[39] with minor modifications to examine cell proliferation as follows: MCF-7 cells were plated in phenol-red-free RPMI1640 medium supplemented with 10% DCC-FBS in 24-well plates at a density of 10^4 cells per well. The cells were treated with 10 nM E_2 or 10 pM−1 μM BFA for 3 days after incubation at 37 °C under 5% CO_2 for 3 days. The cells fixed with 10% cold trichloroacetic acid (TCA) at 4 °C for 30 min were stained with 0.2 mL of 0.4% SRB dissolved in 1% acetic acid for 20 min. The bound protein was solubilized with 0.2 mL of 10 nM unbuffered Tris base and transferred into 96-well plates for the measurement of OD_{490}. Six independent assays were performed for each treatment, and the data were analyzed by the *t*-test.

DNA Microarray Assay. MCF-7 cells were maintained in phenol-red-free RPMI 1640 medium (Invitrogen) containing 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS, Invitrogen) and incubated for 3 days at 37 °C in an atmosphere of 5% CO_2. Aliquots of E_2 (10 nM) and BFA (100 pM) were added individually to the medium at the indicated concentrations and incubated for 3 days. Cells treated with 0.1% DMSO (vehicle) were used as a control. RNA

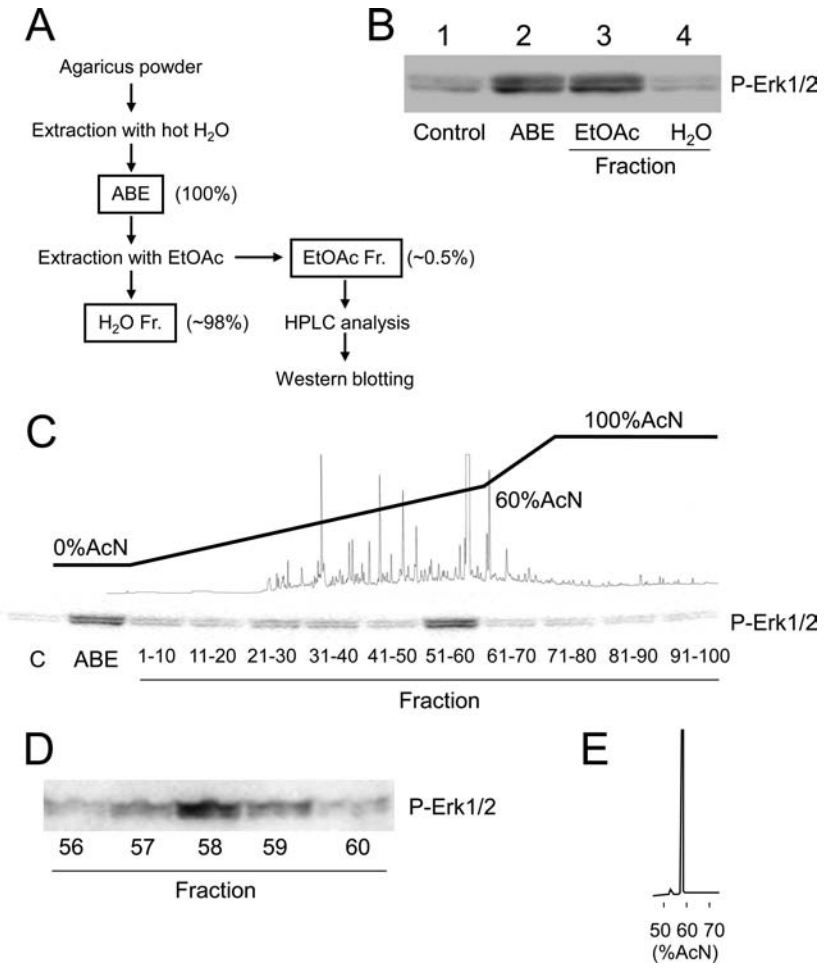

Figure 1. Purification of an Erk1/2-activating component from the extract of *A. blazei* mycelia. (A) Purification scheme. The yields of H_2O and ethyl acetate fractions are indicated in parentheses. ABE, *A. blazei* mycelium extract; EtOAc, ethyl acetate. (B) Extraction with ethyl acetate. Western blot data for P-Erk1/2 are shown for the cells treated with the fractions before (lane 2) or after the extraction with ethyl acetate (lanes 3 and 4 for the ethyl acetate or aqueous fractions, respectively), or the cells without treatment (lane 1). (C) Purification by HPLC. An elution profile of HPLC using the ethyl acetate fraction and the fractions with Erk1/2-activating activity are shown. Western blot data for P-Erk1/2 are shown for the HPLC fractions along with the data for the control cells (C) or the cells treated with ABE. (D) Identification of the active fraction. Western blot data for P-Erk1/2 are shown for the fractions 56−60 in panel C. (E) Result of rechromatography. Fraction 58 was rechromatographed, and the OD_{210} for the elution at 50−60% AcN is shown.

preparation and cDNA labeling followed by focused oligonucleotide-DNA microarray assays were performed as described previously.[18] Briefly, the signal intensity was averaged among duplicated spots, and the ratio of the mean signal intensity for the chemically treated sample to that for the untreated sample was calculated for each gene. The ratios of signal intensity for all genes were normalized against the mean ratio for the 28 control genes. The control genes were selected from housekeeping genes and the genes that did not change their expression levels after treatment with estrogen in MCF-7 cells.[40] Normalized ratios were $\log_2$-transformed and used for correlation analysis. A coefficient of correlation between gene expression profiles was calculated based on linear regression using SPSS 12.0J (SPSS Japan, Tokyo, Japan). Although the oligo-DNA microarray contained a total of 203 genes consisting of 172 estrogen-responsive genes, we used a total of 150 genes selected as highly reproducible estrogen-responsive genes as described before.[18] The microarray data for two independent assays are available in the Gene Expression Omnibus database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE33851.

The UniGene names of the 150 genes obtained from the Entrez database (www.ncbi.nlm.nih.gov) and their functional categories (in parentheses) are as follows: *ACO2, RPS6KA3, PSAT1, CTSD, ENO2, PHGDH, GFPT1, PCK2, FBP1, SFTPB, PMPCA, ASNS, ENO3, ASS, MTHFD2, CDIPT, GOT1, WARS, SHMT2, CPT1A, DHCR24, SORD, FUT8* (enzyme); *ILK, STC2, ULK1, AGTR1, LGALS3BP, NPY1R, PCSK6, EDN2, PTPN18, PVR, CDKN1A, TFF1, PRKCD, ERBB2, GDF15, ARNT2, PIK3C3, ARHGDIA, CTNND2, PRKCSH, IGFBP5* (signaling); *TACSTD2, CCNA1, FTH1, KLF10, AREG, LAMP3, IGFBP4, AREG, ISG20, CAPNS1, TP53I11, PMP22, TSPAN1, MGP, PDZK1, IFRD1* (proliferation); *ENO1, GTF2I, EGR3, CEBPB, FOS, ATF3, NRIP1, TCEA1, ATF3, TCEA1* (transcription); *CLIC4, LCN2, TCN1, CDH18, SLC1A5, SLC1A4, SLC7A5, SLC7A11, TSEN15, S100P (1), EFEMP1, S100P (2), IMP4, GARS, RCN1, SLC12A2* (transport); *RAP1GAP, VAMP5, NCOA3, CFB, KRT8, SH3BGR, AKR1C4, YARS, C4orf27 (1), CDC14B, MAL, EIF3B, FOSL2, HSPA1A, HDAC6, PMAIP1, H3F3B, SLC26A3, CYP19A1, SELENBP1, NCKAP1, HSD17B2, CDC6, STS, FOSL2, MATN2, TMEM109, TPD52L1, EFTUD2, HAX1, GUCA2B, JUN, CCR2, IL2RB, RBBP8, CDSN, KIAA0196, ADORA2A, SH3BGR, Hs3BAC, C19orf21, ESR2, BCL2L11, C4orf27 (2), Hsc17, APPL1, NCOA1, BSN, IL1R1, RACGAP1, DAZAP2, IER3, DDEF1, DHX29, TRIB3, HSP90B1, BRCA1, HSPA5, HMMR, SECTM1, CBX1, QSOX1, CaMK2N1, TM4SF1* (others).

Real-Time RT-PCR. MCF-7 cells were cultured as described above. Total RNA was isolated using an RNeasy Plus Mini kit (QIAGEN K. K., Tokyo, Japan). The first-strand cDNA was synthesized from 1 μg

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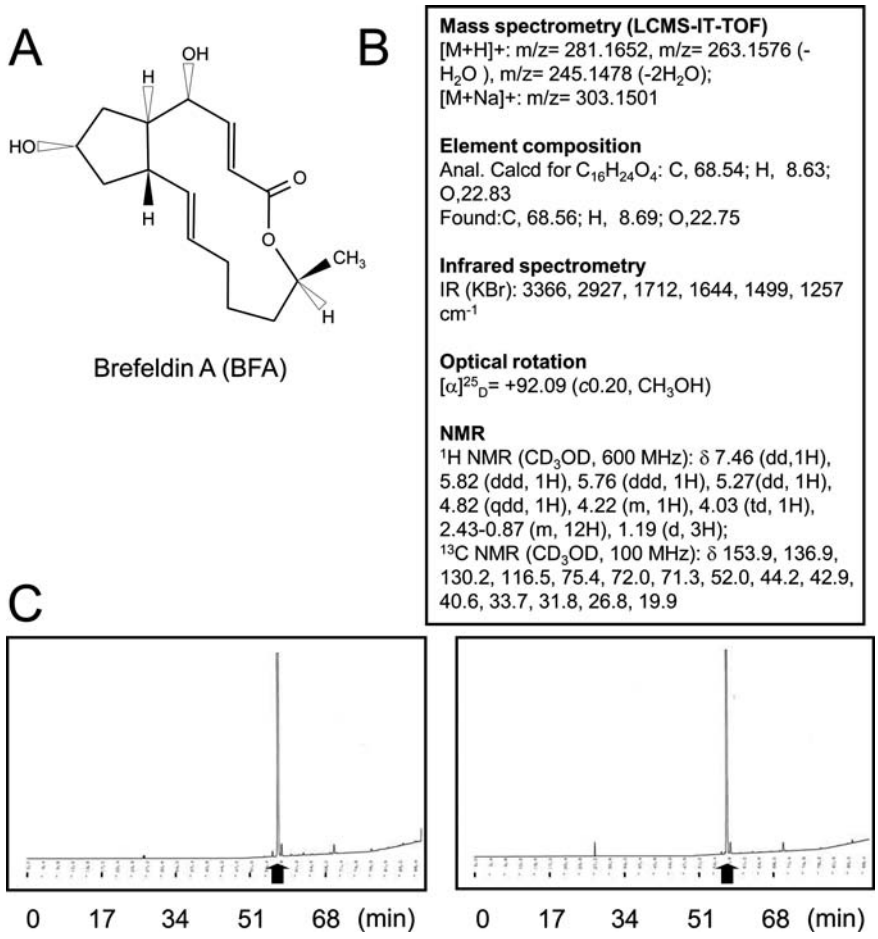

Figure 2. Identification of the Erk1/2-activating component in the extract of *A. blazei* mycelia. (A) Structure of brefeldin A (BFA). (B) Summary of the data used for identification. The purified material was examined by mass spectrometry, elemental composition analysis, optical rotation analysis, and NMR spectrometry. OD_{210} (top) or Western blot (bottom) data for ABE and HPLC fractions are shown. AcN; acetonitrile. (C) Comparison of the purified component with BFA from *Eupenicillium brefeldianum* by HPLC. The purified component (0.5 mg) mixed with BFA (purified from *Eupenicillium brefeldianum*, 0.5 mg, left) or BFA alone (0.5 mg, right) was analyzed by HPLC. Arrows indicate the positions of BFA.

of total RNA using SuperScript III (Invitrogen). Real-time RT-PCR was performed with FastStart DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN) using a LightCycler (Roche Applied Science) under the reaction consisting of denaturation at 95 °C for 3 min and 40 cycles of 95 °C for 10 s and 60 °C for 20 s. *β-Actin* gene was used as an internal control. The ratio of the amount for each specific gene against that of *β-actin* was calculated and used for quantification. Real-time RT-PCR was performed two to three times for each gene using the following primers: 5′-CAAAGGACTGGTGTCAAAGATG-3′ and 5′-AGATGTGGTGCATCCTGTCTGTT-3′ for *RPS6KA3*, 5′-GAGGAAATCATTGAGCCATACAG-3′ and 5′-GAGCACTAGACACAGCACCTTTT-3′ for *SLC12A2*, 5′-TTGTGGTTTTCCTGGTGTCA-3′ and 5′-CCGAGCTCTGGGACTAATCA-3′ for *TFF1*, 5′-CTGGAACGGTGAAGGTGACA-3′ and 5′-AAGGGACTTCCTGTAACAACGCA-3′ for *β-actin*.

RESULTS

Purification and Identification of an Erk1/2-Activating Component in the Extract of *A. blazei*. We previously found that an extract of *A. blazei* mycelia (ABE) has estrogenic activity and activates Erk1/2.[18] As the extract clinically showed preventive effects on atherosclerosis and atherosclerosis is partly associated with a lack of estrogen, the component with estrogenic activity could potentially be used for the treatment of atherosclerotic symptoms. Therefore, in this work, we explored the identity of an essential component for estrogenic activity. Because ABE did not show any growth-stimulating activity in breast cancer MCF-7 cells, it might act as a selective estrogen-receptor modulator (SERM). SERMs are a category of estrogenic chemicals that differ from natural estrogen in target-tissue specificity and agonistic/antagonistic activity and include a variety of phytoestrogens and xenoestrogens.[41,42] The agonist/antagonist activity of SERMs is attributed to the F-domain of ERs whose function is not well-defined.[43] As the rapid activation of Erk1/2 is an important indicator of estrogenic activity, we started to purify the component by examining the activation of Erk1/2. After several attempts, we established a scheme for the purification of the component (Figure 1A).

Dried powder of *A. blazei* mycelia (*Agaricus* powder) was first extracted with hot water by boiling for 30 min. The extract of *A. blazei* (ABE) was then treated with ethyl acetate, giving H_2O (~98% yield in dry weight) and ethyl acetate (~0.5% yield) fractions. As the ethyl acetate fraction contained the Erk1/2-activating component (Figure 1B, lane 3), it was further analyzed by HPLC (Figure 1C). We found that the Erk1/2-activating component was eluted at 58 min (58% v/v acetonitrile) into a single fraction (fraction 58) (Figure 1C,D). The fraction examined by rechromatography showed a single peak (Figure 1E), indicating that it most likely

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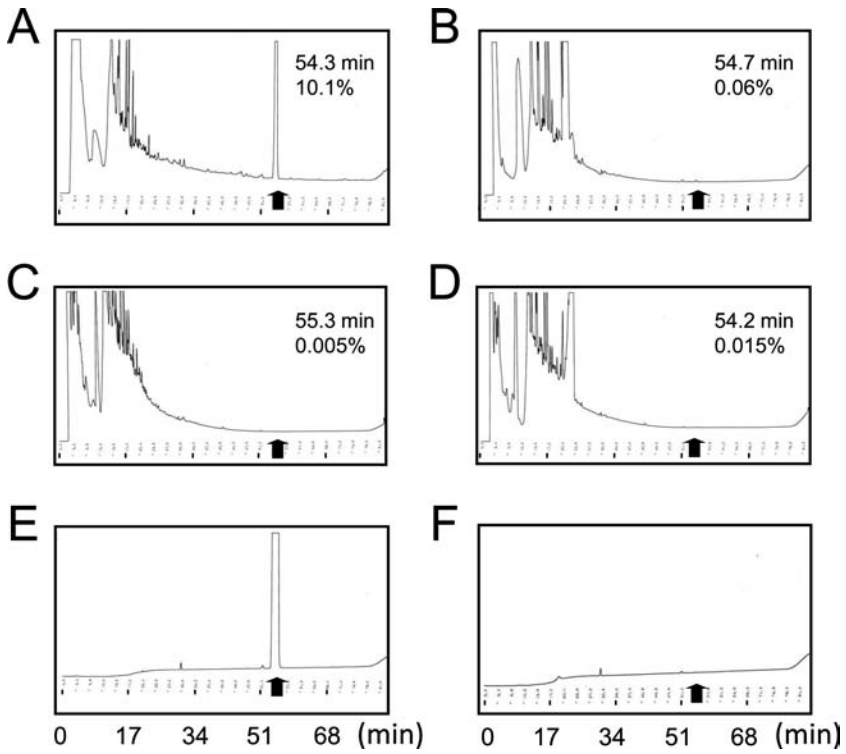

Figure 3. Identification of BFA in the mycelium but not fruiting body of *A. blazei*. HPLC was performed with (A) the extract of the mycelium, (B–D) extracts of the fruiting body obtained from three different commercial sources, (E) BFA, or (F) vehicle. The amounts of materials at the indicated positions (in percentages) are shown in panels A–D. Arrows indicate the positions of BFA.

contained a single component. Thus, the dry weight (400 mg) of 8 mL of ABE solution gave 2 mg (dry weight) of the ethyl acetate fraction, indicating that the concentration of BFA in ABE solution was 0.25 mg/mL and the amount of the purified material in *Agaricus* powder was 0.8 mg/g of powder.

The component was identified by mass spectrometry, ^{1}H and ^{13}C NMR spectrometry, optical rotation analysis, elemental composition analysis, and infrared spectrometry as brefeldin A (BFA) (Figure 2A,B). To again confirm that the purified component behaves similarly to BFA on HPLC, the BFA from *Eupenicillium brefeldianum* was used as a marker (Figure 2C). As both materials showed peaks at exactly the same position, we concluded that the component was BFA.

Presence of Brefeldin A in the Mycelium but Not Fruiting Body of *A. blazei*. Dried *A. blazei* is widely available commercially as dried mushrooms or powder of mushrooms because cultivation of the mushroom or the fruiting body of *A. blazei* is relatively easy. Moreover, as the mycelium is usually very small, culturing in liquid medium is necessary, which is time-consuming. However, the components, and their effects, are expected to differ. We therefore examined whether BFA found in the mycelium was also present in the fruiting body of *A. blazei* (Figure 3). BFA was not detected in fruiting bodies obtained from three different sources (Figure 3B–D). So, BFA is specifically found in the mycelium of *A. blazei*.

Estrogenic Activity of Brefeldin A. Finally, we examined the estrogenic activity of BFA by DNA microarray assay (Figure 4). The assay was performed to examine gene expression profiles for E_2 and 100 pM BFA using a set of 150 estrogen-responsive genes.[44] A correlation coefficient (or *R* value) of 0.61 was obtained for the profiles (Figure 4A), indicating a modest but distinct correlation between E_2 and 100 pM BFA. Among the genes examined, *MGP* (functional category proliferation), *IFRD1* (proliferation), *SHMT2* (enzyme), *SORD* (enzyme), *GTF2I* (transcription), *PRKCSH* (signaling), *SLC12A2* (transport), *RCN1* (transport), and *IER3* (others) showed significant (greater than 2-fold degree of change) response to BFA. We then examined three genes by real-time RT-PCR (Figure 4B), whose expression levels showed similar (*RPS6KA3* and *SLC12A2*) or different (*TFF1*) changes between the treatments with E_2 and 100 pM BFA in the DNA microarray assay (Figure 4A). The results showed that BFA up-regulated the expression of *RPS6KA3* and down-regulated that of *SLC12A2*. In contrast, *TFF1*, which is a well-known marker for E_2-induced gene expression, did not show a significant change upon treatment with BFA.

BFA-Induced Signal Transduction. As was observed for ABE,[18] BFA did not have any growth-stimulating effect on MCF-7 cells (Figure 5A). This prompted us to investigate the difference in the signaling pathways between E_2 and BFA (Figure 5B). BFA stimulated Erk1/2 as expected, but unlike for E_2, there was no inhibition of Erk1/2-stimulating activity with an ER antagonist, ICI 182,780, suggesting the involvement of additional pathways, especially at or around the receptor. In contrast, the activities of Akt and P70S6K (a target of mTOR) were stimulated by the treatment with both E_2 and BFA, and the stimulations were inhibited with a PI3K inhibitor (LY 294002) or a mTOR inhibitor (rapamycin), suggesting some similarity in their pathways once the signaling is initiated.

DISCUSSION

Herein, we reported for the first time that brefeldin A (BFA) has estrogenic activity. BFA is a lactone antibiotic produced by *Eupenicillium brefeldianum* and other fungi. BFA was initially isolated as an antiviral antibiotic[45] but is now primarily used in the study of protein transport because of its ability to inhibit the

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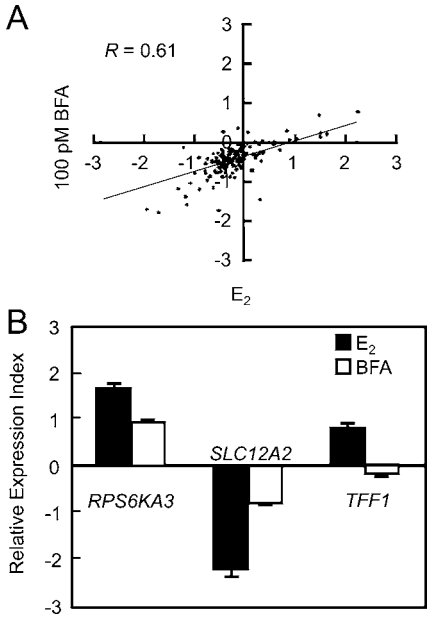

Figure 4. Correlation of gene expression profiles between E_2 and BFA. Expression profiles of 150 estrogen-responsive genes were examined by DNA microarray assay using mRNA from MCF-7 cells after treatment with 10 nM E_2 or 100 pM BFA. The axes give $\log_2$-transformed ratios of the fluorescent intensity for the cells treated with a chemical to that without the chemical (control) for each treatment. A coefficient (*R* value) for the correlation between the two profiles was calculated based on linear regression. A result representative of two independent assays is shown. (B) Real-time PCR analysis for the expression of *RPS6KA3*, *SLC12A2*, and *TFF1*. The expression level of a gene was normalized to that of *β-actin*, and the $\log_2$-transformed value (relative expression index) is shown as the mean ± SD of the data from two (*TFF1*) or three (*RPS6KA3* and *SLC12A2*) independent experiments.

transport of proteins from the endoplasmic reticulum to the Golgi apparatus and to induce retrograde protein transport from the Golgi to endoplasmic reticulum.[46] In mammalian and yeast cells, the main targets of BFA are Arf-family proteins, which are small GTPases involved in the formation of transport vesicles by recruiting coat proteins to intracellular membranes.[47]

Because of a lack of growth-stimulating activity (Figure 5A), there was no efficient way to detect estrogenic activity of BFA. We first suspected that the growth-stimulating effect of ABE on MCF-7 cells was masked by the growth-suppressive activity of a different component.[18] It turned out that the purified component, BFA, showed an estrogen-like gene expression profile but lacked growth-stimulating activity. The DNA microarray analysis allowed us to examine the activity at the gene expression level, and even without explicit activity at the cellular level, we were able to detect intracellular activities (Figure 4, ref 18). Accordingly, we found a group of chemicals in this category and reported BFA here as the first among them. These chemicals could be collectively termed "silent estrogens", a type of chemical characterized by estrogenic gene-expression profiles and some cell functions, such as receptor-mediated signaling, but explicitly lacking cell growth-stimulating activity.

The nature of silent estrogens could have arisen from their unique characteristics not only at the cellular level but also at the molecular level. Although the mechanism of action at the molecular level remains to be examined, one can assume that the lack of growth-stimulating activity is due to its structure. BFA is a relatively small molecule; its molecular weight (MW) of 280 is comparable to that of a typical estrogen 17β-estradiol (MW 272) and a phytoestrogen, genistein (MW 270), although BFA is less hydrophilic and lacks a phenolic hydroxyl group. Thus, there might be a difference in its interaction with ERs, particularly in the way it affects the activation functions attributed to the F-domain of ERs as found in some SERMs or in the interaction with other receptors such as GPCRs. The effect of ICI 182,780 on P-Erk1/2 was similar to that shown in Figure 5B, as observed for GPR30, a GPCR, for the binding of another estrogenic chemical, biophenol A.[22] On the other hand, because BFA is an inhibitor of protein transport, there could be a separate mechanism of specific inhibition of the transport of cell-growth-related proteins.

We adopted a strategy of purifying BFA from ABE as a rapid Erk1/2-activating component because of the simplicity of the assay (Figure 1). The assay is based on the rapid activation of Erk1/2, as revealed by the increase in the amount of phospho-Erk1/2 (P-Erk1/2), and thus saves on time and expense while providing a clear result, as signaling occurs within a few minutes before activating many other signaling pathways. From the examination of signaling pathways, it is likely that a significant difference occurred at or around the receptor. There was a clear difference in the effect of an ER antagonist, ICI 182,780, between E_2 and BFA (Figure 5B). We also observed a difference in the behavior of some target genes for the estrogen-induced expressional regulation such as *TFF1* (Figure 4B). *TFF1* or *pS2* is known to have roles in differentiation and tumor growth,[48,49] and the lack of stimulation of *TFF1* might be related to a lack of such cellular activities. In contrast, there was some similarity as shown in the PI3K/Akt/P70S6K pathway (Figure 5B), which is a major estrogen signaling pathway.

We also found that BFA was present in the mycelium but not fruiting body of *A. blazei* (less than 0.1% in HPLC fractions, Figure 3). This is rather surprising, because many applications of *A. blazei* have been based on the use of mushrooms or the fruiting body, presumably because of ease of cultivation. To harvest mycelia, a liquid culture system is required together with a method of efficiently extracting the ingredients within fungal cells. Therefore, there are few examples of the supplementary use of mycelia in traditional medicines and functional foods, and the use of mycelia has been mostly limited to storage and the production of some polysaccharide ingredients.[50] Therefore, the study of the use of the mycelium and its active components, such as reported here, is important.

In our previous study, ABE exhibited a positive effect on atherosclerosis, as revealed by preliminary clinical observations such as an improvement of the OxLDL level in hyperlipidemic patients and improvement of experimental atherosclerotic lesions in rabbits fed a high-cholesterol diet after the uptake of ABE, and we reasoned that these findings are due to its estrogenic activity.[18] In a preliminary examination, however, we found that, unlike *Agaricus* powder or an *Agaricus* extract (ABE), BFA did not significantly show activity for the improvement of atherosclerotic lesions (Supplementary Figure 1, Supporting Information). The solubility of BFA in water is 2.8 mg/mL, and its poor solubility is an obstacle against the development of drugs.[51] Therefore, various efforts have been made to enhance its solubility including chemical modification to directly improve its solubility[51] and genetic selection of bacteria to increase its concentration.[52] This problem is not

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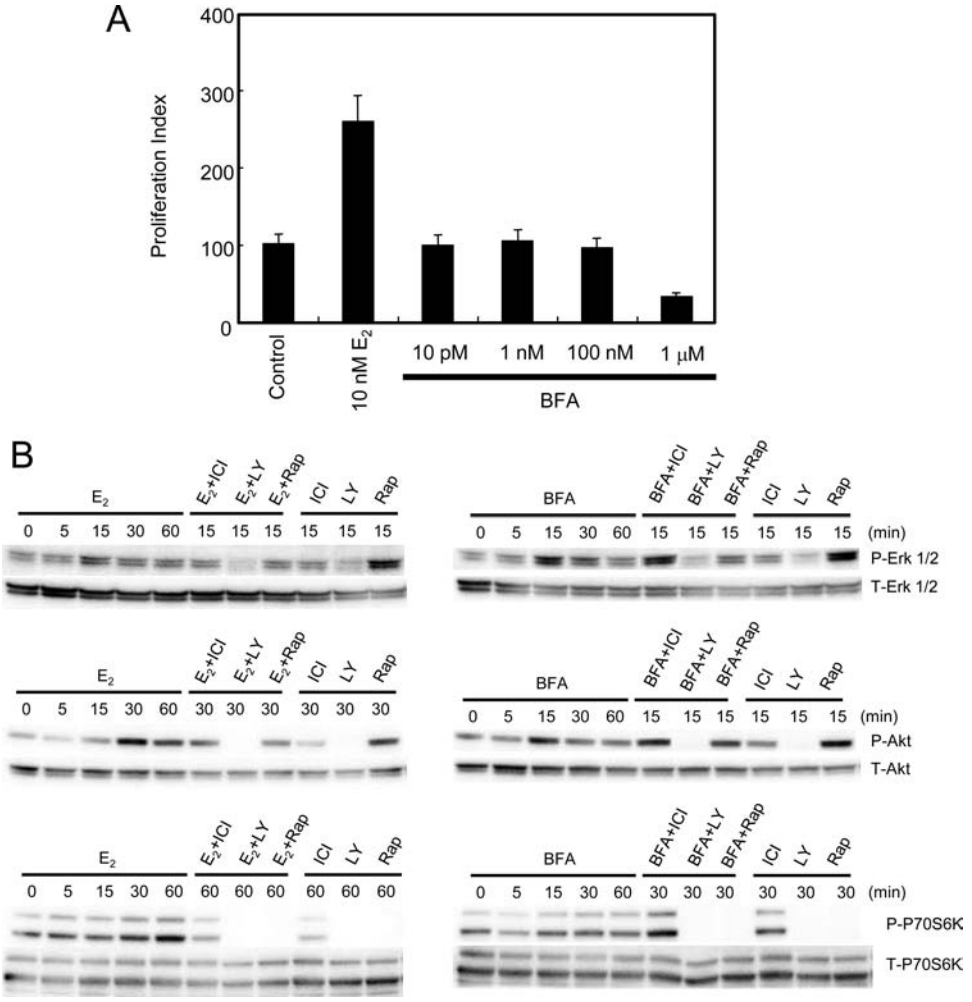

Figure 5. Cell-growth assay and Western-blot analysis of BFA-treated cells. (A) Cell-growth assay. Growth-stimulating activity was examined by SRB assay for 10 nM estrogen (17β-estradiol) or the indicated concentrations of BFA. Cells treated with vehicle (DMSO) were used as a control, and the value relative to the control is expressed as a proliferation index. (B) Western-blot analysis of active/total Erk1/2, Akt, or P70S6K. MCF-7 cells were cultured with 10 nM of E_2 or BFA in the presence or absence of the indicated inhibitor, ICI 182,780 (ICI), LY 294002 (LY), or rapamycin (Rap), for the indicated periods of time (minutes), and cell extracts were subjected to Western-blot analysis for phosphorylated (P-) or total (T-) proteins as indicated.

limited to BFA alone. A roughly equivalent solubility has been known to glycyrrhetic acid (1.18 mg/mL),[53] which is produced by intestinal digestion of glycyrrhizin, an active component in licorice (*Glycyrrhiza glabra*) widely used in foods and medicinal products. Despite its efficacy against inflammation, virus, and tumors, its therapeutic use is limited because of its poor solubility.[54] Therefore, poor solubility is actually a common and serious problem for these chemicals in their direct use as pure drugs, whereas solubility is not a major problem for the use of these natural products probably because other components might help absorption or inhibit degradation. Estrogen and many estrogenic chemicals are hydrophobic and thus need proper carriers such as sex-hormone-binding protein or proper modifications such as glycosidation to increase their solubility. Thus, it might be reasonable to see that BFA alone did not show apparent activity of improving atherosclerosis in rabbits, although the activity was observed in ABE (Supplementary Figure 1B, Supporting Information), and more careful examinations are needed to evaluate the effect of BFA in animals.

Estrogen is known to lower the risk of atherosclerosis and has been used to treat atherosclerotic patients. However, as estrogen stimulates cell growth, it might increase the risk of cancer. Therefore, the study of silent estrogens shown here will open a new pathway not only for exploring the therapeutic/supplementary/pharmacological use of ABE but also for screening and characterizing a new type of chemical along with new signaling pathways.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods and results for animal experiments and figure showing the effect of BFA on atherosclerotic lesions in coronary arteries in rabbits. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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