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Antiestrogenic Effects of Marijuana Smoke Condensate and Cannabinoid Compounds

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The antiestrogenic effects of marijuana smoke condensate (MSC) and three major cannabinoids, *i.e.*, Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN), were evaluated using *in vitro* bioassays, *viz.*, the human breast cancer cell proliferation assay, the recombinant human estrogen receptor (ER) competitive binding assay, and the reporter gene assay. The inhibitory effects on estrogen were also examined using the ethoxyresorufin-*O*-deethylase (EROD) assay, the aromatase assay, and the 17β-estradiol (E_2) metabolism assay. The results showed that MSC induced the antiestrogenic effect *via* the ER-mediated pathway, while THC, CBD, and CBN did not have any antiestrogenic activity. This suggests that the combined effects of the marijuana smoke components are responsible for the antiestrogenicity of marijuana use. In addition, MSC induced the CYP1A activity and the E_2 metabolism, but inhibited the aromatase activity, suggesting that the antiestrogenic activity of MSC is also related to the indirect ER-dependent pathway, as a result of the depletion of the *in situ* E_2 level available to bind to the ER. In conclusion, pyrogenic products including polycyclic aromatic hydrocarbons (PAHs) in the non-polar fraction, which is the most biologically active fraction among the seven fractions of MSC, might be responsible for the antiestrogenic effect.

Key words: Marijuana, Cannabinoids, Drug abuse, Antiestrogenic effects, Polycyclic aromatic hydrocarbons (PAHs)

INTRODUCTION

Marijuana (*Cannabis sativa*) is one of the most frequently abused drugs in Korea second only to methamphetamine (Chung *et al.*, 2004). It has been traditionally cultivated to produce hemp clothes and grows naturally in some districts. Therefore, it has become the drug first abused for recreational purposes in Korea. In oriental medicine, cannabis seeds without the skin, which do not contain any hallucinogenic compounds, have been used to relieve constipation. Usually a dry, shredded green and/or brown mix of flowers, stems, seeds, and leaves of the hemp plant is smoked to induce the hallucinogenic effects.

Marijuana contains more than 400 compounds, among which the terpene chemicals unique to the plant genus *Cannabis* are called cannabinoids. The main cannabinoids are Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN), with THC being the most potent psychoactive compound in marijuana. The hallucinogenic action of cannabinoids is mainly mediated by two cannabinoid receptors (CB_1, CB_2) in the central nerve system and certain peripheral tissues.

It is known that the chronic use of marijuana can have adverse effects on the endocrine and reproductive systems. In particular, studies with laboratory animals and retrospective studies on women who had abused marijuana revealed a decrease in the level of the sex steroid hormones and a disruption of ovulation. In addition, cannabinoids disturb the sexual maturation of prepubertal females (Rosenkrantz and Esber, 1980; Smith and Asch, 1984; Ashton, 1999).

The antiestrogenic effect of marijuana use is similar to that of cigarette smoking (Berstein *et al.*, 1998; Spangler, 1999; Mueck and Seeger, 2005). Besides cannabinoids, marijuana smoke contains various pyrogenic products including polycyclic aromatic hydrocarbons (PAHs) that are similar to those contained in tobacco smoke including naphthalene, 1-methyl naphthalene, 2-methyl naphthalene, benzo(a)anthracene, and benzo(a)pyrene, but at greater amounts than in tobacco (Robert *et al.*, 1988; Roth *et al.*,

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2001). Some PAHs display antiestrogenic properties, which are mediated *via* cross-talk between the estrogen receptor (ER) and the arylhydrocarbon receptor (AhR) (Chaloupka *et al.*, 1992; Tran *et al.*, 1996; Arcaro *et al.*, 1999).

The relationship between cannabinoids or crude marijuana plant extracts and the ER has been investigated regarding the adverse effects of marijuana (Rawitch *et al.*, 1977; Smith *et al.*, 1979; Sauer *et al.*, 1983; Ruh *et al.*, 1997; Watanabe *et al.*, 2005). In recent studies, cannabinoids, such as THC and CBD, could not stimulate the ER (Ruh *et al.*, 1997), while crude marijuana plant extracts caused the proliferation of human breast cancer cells (Watanabe *et al.*, 2005). However, the interactions between marijuana smoke condensate (MSC), to which marijuana smokers are actually exposed, and the ER as well as its antiestrogenic effect remains undetermined.

Therefore, this study examined the antiestrogenicity of MSC and compared this with those of the potent psychoactive compounds of marijuana, *i.e.*, THC, CBD, and CBN. MSC was further fractionated using liquid-liquid partition and column chromatography to identify the biologically active fractions. The mechanism of its action through the ER was also investigated in order to determine the relation between recreational marijuana smoking and the adverse effects of an estrogen imbalance.

MATERIALS AND METHODS

Chemicals

THC, CBD, and CBN were obtained from Cerilliant (TX, U.S.A.) and 0.1 M stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, U.S.A.). 17β-Estradiol (E_2), tamoxifen (TM), an estrogen receptor antagonist, and 4-hydroxyandrostenedione (HA) were purchased from Sigma-Aldrich, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Chem Service (PA, U.S.A.).

Cell culture

The human breast cancer cell line, MCF7-BUS, which is estrogen sensitive, was kindly provided by Dr. Soto (Tufts University, MA, U.S.A.). MCF-7 (human breast cancer cells), H4IIE (rat hepatoma cells), and JEG-3 (human placental cells) cells were obtained from the American Type Culture Collection (VA, U.S.A.). The cells were grown in DMEM supplemented with 5% FBS in a humidified incubator at 37°C in a 5% CO_2 and 95% air atmosphere.

Preparation of MSC

Non-filtered marijuana cigarettes were prepared from dried marijuana plants (National Institute of Scientific Investigation, Korea) using a manual cigarette-stuffing machine (Privileg, Germany). Twenty marijuana cigarettes (average weight, 646 mg) were burned and MSC was collected on a single fiber filter (10×10 cm, Sibata, Japan) in a smoking chamber using a high-flow vacuum pump connected to a cascade impactor (Dusturbo, Korea). The dry weight of MSC was measured. The twenty non-filtered marijuana cigarettes generated a total of 339 mg of MSC. MSC was extracted by sonication with methylene chloride, filtered, and evaporated to dryness. For the *in vitro* bioassays, it was dissolved in DMSO at a concentration of 100 mg/mL and stored at -80°C until needed. It was diluted with DMSO to the desired concentrations for the treatment of the cells.

Fractionation of MSC

MSC was fractionated using an acid-base liquid-to-liquid partition sequence and silica gel column chromatography according to the method reported by Lewtas *et al.* (1990) as follows (Fig. 1). In summary, the dried MSC extract was redissolved in methylene chloride and partitioned into basic (F1), acidic (F2), and neutral (F3-F7) fractions. The neutral fraction was further subfractionated using silica gel column chromatography into following fractions: F3 was eluted with hexane, F4 with a 1:1 mixture of methylene chloride and hexane, F5 with methylene chloride, F6 with a 1:1 mixture of methylene chloride and methanol, and F7 with methanol. Each fraction was collected separately, evaporated to dryness, and dissolved in DMSO at a

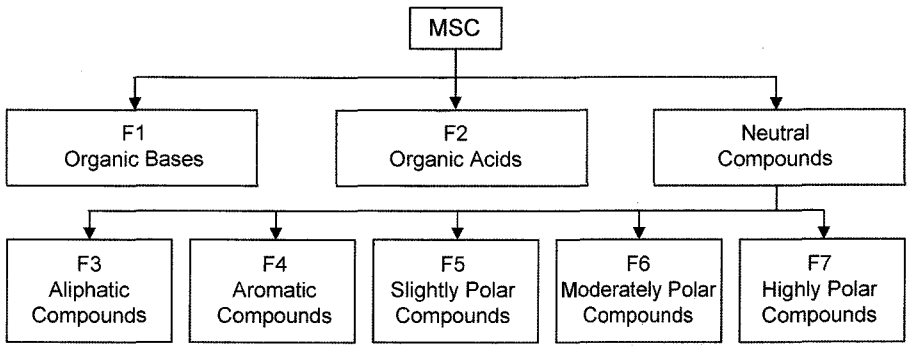

Fig. 1. Scheme of the fractionation procedure of MSC

concentration of 100 mg/mL and stored at -80°C until needed. Each sample was diluted to the desired concentrations with DMSO.

Cell proliferation assay

The cell proliferation assay using MCF7-BUS cells was carried out as described elsewhere (Perez *et al.*, 1998). Briefly, the cells were harvested with 0.05% trypsin-0.53 mM EDTA · 4Na and resuspended in 5% FBS-DMEM. They were seeded onto 48 well plates at a density of 5×10^5 cells/well, and incubated in a 5% CO_2 and 95% air atmosphere at 37°C for 48 h. The cells were then treated with a medium containing MSC, THC, CBD, or CBN at the indicated concentrations along with 10^{-11} M E_2 in phenol red free 10% CDFBS-DMEM. After the cells were incubated for 144 h, the Sulforhodamine B (SRB) assay was carried out to evaluate the extent of cell proliferation. The relative proliferation effect (RPE) was calculated using the following equation: RPE = [(S-1)/(E-1)] × 100, where S = the proliferation of the samples, and E = the proliferation of a positive control (E_2). The biologically active fractions were identified by carrying out another cell proliferation assay with the fractionated samples in the same way.

Competitive ER binding assay

The competitive ER binding assay was performed as described earlier (Arcaro *et al.*, 1999). Briefly, 1.5 nM of recombinant human ER was incubated with MSC, THC, CBD, CBN, or E_2 in the presence of 2.5 nM of [2,4,6,7-^{3}H] E_2 at room temperature for 4 h. Subsequently, 100 μL of a 50% (v/v) hydroxyapatite slurry was added to the reaction mixture. The hydroxyapatite-bound receptor-[^{3}H] E_2 complex was separated by centrifugation at 200×g for 20 min and the radioactivity of the pellet was determined using a liquid scintillation counter (Pharmacia LKB, NY, U.S.A.). The amount of the receptor bound-[^{3}H] E_2 in the presence of the indicated concentrations of the compounds was calculated after correcting for any non-specific binding as determined by the amount of the receptor-bound [^{3}H] E_2 in the presence of a 200-fold excess of E_2. The data is expressed as the ratio of the receptor-bound [^{3}H] E_2 in the presence of the compounds to 0.1% DMSO used as a control.

Reporter gene assay

The reporter vector was constructed by inserting two estrogen response elements (EREs) of Vitellogenin A2 into the pGL3 promoter vector. MCF-7 cells suspended in phenol red free 3% CDFBS-DMEM were seeded onto 48 well plates at a density of 1×10^5 cells/well, and incubated in a 5% CO_2 and 95% air atmosphere at 37°C for 24 h. The cells were washed with OPTI-MEM, and 0.5 mL FBS free OPTI-MEM I was then added. 200 μL of the FBS free OPTI-MEM I medium containing 2 μg of pERE2-Luc and 4 μg of LipofectAMINE was added to each well after incubating the medium at 37°C for 30 min. The cells were incubated in a 5% CO_2 and 95% air atmosphere at 37°C for 10-15 h. The cells were treated with the indicated levels of the compounds along with 10^{-11} M E_2 in phenol red free 5% CDFBS-DMEM and incubated again for 24 h.

For the luciferase assay, the cells were washed with PBS and incubated for 10 min with a lysis buffer. The cell lysate was centrifuged and the luciferase activity in the upper layer was measured using a Turner design luminometer TD-20/20 (Promega, WI, U.S.A.), after adding 20 μL of a Luciferase assay reagent. The protein level was then measured using the Bradford method (Bradford, 1976) with BSA as the standard.

Ethoxyresorufin-O-deethylase (EROD) assay

The EROD assay was performed using the H4IIE cell line using a slight modification of a method described elsewhere (Drenth *et al.*, 1998). The cells were plated onto 96 well plates at a density of 3×10^4 cells/well in MEM and incubated in a 5% CO_2 and 95% air atmosphere at 37°C. After 2 days, when the wells reached approximately 80% confluence, the cells were exposed to the indicated concentrations of the compounds in phenol red free 5% CDFBS-DMEM, and incubated for 48 h. 4 μM ethoxyresorufin and 5 μM dicumarol in DMEM were then added after washing the cells twice with DMEM. After incubating the cells at 37°C for 30 min, the enzymatic conversion of ethoxyresorufin to resorufin was evaluated. The protein level was measured using the Bradford method (Bradford, 1976) with BSA as the standard. The biologically active fractions were identified using another EROD assay with the fractionated samples in the same way.

E_2 metabolism assay

Radiometric analysis was used to assess the ability of the compounds to increase or inhibit the catabolism of E_2 using a slight modification of the method described in a previous study (Arcaro *et al.*, 1999). MCF7-BUS cells were seeded onto 24 well plates and exposed to each compound. After incubating for 72 h, 2 mL of the medium was removed from each well, mixed with 0.2 mL of 10 nM [2,4,6,7,16,17-^{3}H] E_2, and returned to the cells. The medium was then collected 24 h later, and the [^{3}H] E_2 was separated by adsorption onto charcoal. The level of radioactivity from the tritium recovered in the medium in the form of tritiated water was determined using a liquid scintillation counter. The amount of aqueous tritium is an indication of the degree of the E_2 metabolism at any or all of the [^{3}H]-substituted positions.

Aromatase assay

The aromatase activity was determined by measuring

the amount of [^{3}H] H_2O released upon the conversion of [1β-^{3}H] androstenedione to estrone, as described elsewhere (Tanaka *et al.*, 1996). Briefly, JEG-3 cells were seeded onto 24 well plates with the growth medium containing 3% FBS. After 24 h, the cells were exposed to the compounds for 24 h and further incubated with [1β-^{3}H] androstenedione for 30 min. The medium was extracted with chloroform and centrifuged. The aqueous supernatant was added to 5 mL of a scintillation fluid, and the radioactivity was evaluated. The cell protein content, where the amount of radioactivity in [^{3}H] H_2O was standardized, was determined using the Bradford method (Bradford, 1976) after being dissolved in 1.0 N NaOH.

Statistical analysis

Each assay was performed at least in triplicate and the data is expressed as a mean ± SD. Statistical analysis was carried out using the Student's *t*-test with SigmaPlot 2000 (Jandel Science Software, CA, U.S.A.) and Excel 2000 (Microsoft, NY, U.S.A.) computer software. A *P*-value < 0.05 ($p<0.05$) was considered significant.

RESULTS

Effects of MSC and the cannabinoid compounds on the antiestrogenicity through ER-ligand binding

The effects of MSC and the major cannabinoids on the human breast cancer cell proliferation, their competitive ER binding abilities, and transcriptional stimulations of the E_2-responsive reporter gene were examined in order to determine if they can act as antiestrogenic compounds through ER-ligand binding. In the cell proliferation assay using MCF7-BUS cells, THC, CBD, and CBN at 10^{-6} M (Fig. 2A) and below (data not shown) had no effect on the cell proliferation induced by E_2, whereas MSC was found to inhibit the cell proliferation at a concentration of 0.1-10 μg/mL (Fig. 2B), which is in the non-toxic concentration range (data not shown). 5 μg/mL and 10 μg/mL MSC significantly reduced the level of cell proliferation induced by E_2, and the corresponding RPEs were 68.2 ± 2.4% and 39.4 ± 3.4%, respectively.

There are two types of ERs, ER-α, and ER-β. The former is involved in reproductive events (Kuiper *et al.*, 1998). Therefore, the competitive binding ability with ER-α was examined to determine if MSC and the cannabinoid compounds bind directly to ER-α. As shown in Fig. 3, MSC significantly inhibited the binding of [^{3}H] E_2 to ER-α by 34.2 ± 0.6% at 5 μg/mL, compared with the control. This indicates that MSC directly interacts with ER-α. However, THC, CBD, and CBN at 10^{-6} M and below did not show any binding affinity (data not shown).

The transcriptional stimulations of the E_2-responsive reporter gene transfected into MCF-7 cells by the

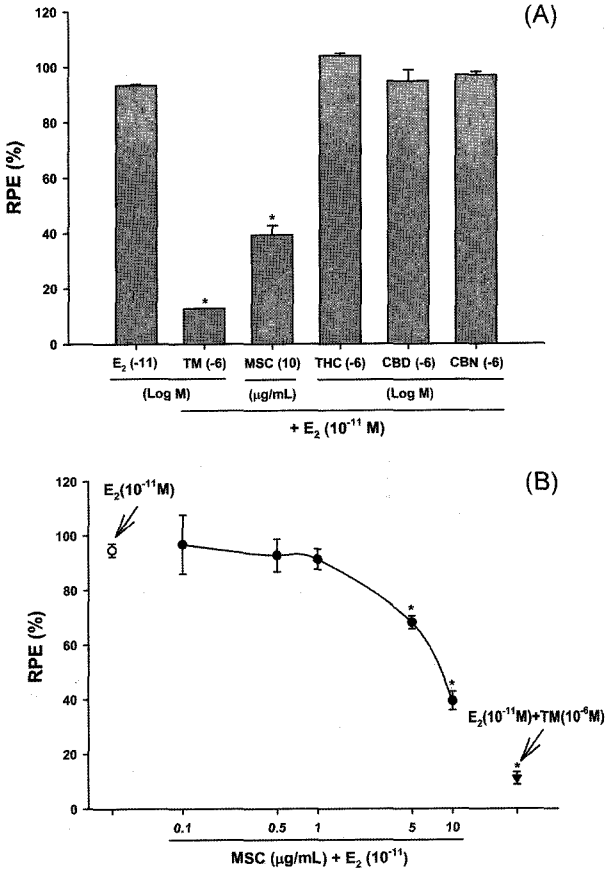

Fig. 2. Effects of MSC, THC, CBD, and CBN on the MCF7-BUS cell proliferation in the presence of E_2. (A) MCF7-BUS cells were treated with the indicated concentrations of E_2 or a combination of E_2 and TM or each compound for 144 h. The SRB assay used to measure the cell proliferation was performed as described in the Methods section. The proliferative effect of the compounds relative to E_2 (10^{-10} M, 100%) is represented by the RPE (Relative Proliferative Effect). The results are expressed as the mean ± SD of three separate experiments. Values significantly different from the control are indicated by an asterisk (*$p<0.05$ by Student's *t*-tests). (B) MCF7-BUS cells were treated with the indicated concentrations of E_2 or a combination of E_2 and MSC at various concentrations (0.1-10 μg/mL) or TM (10^{-6} M) for 144 h.

compounds in the presence of 10^{-11} M E_2 were examined in order to confirm the antiestrogenic activity of MSC and determine if this effect was mediated *via* ER-ligand binding followed by ERE complexation. 0.5-10 μg/mL of MSC decreased the transcription of the E_2-responsive reporter gene induced by E_2, whereas 10^{-6} M THC, CBD, and CBN had no effect (Figure 4A, B). Cannabinoids at concentrations < 10^{-6} M did not show any effects (data not shown).

Effects of MSC and the cannabinoid compounds on the regulation of endogenous estrogen level

The effects of MSC, THC, CBD, and CBN on the metabolism and biosynthesis of E_2 were tested in order to

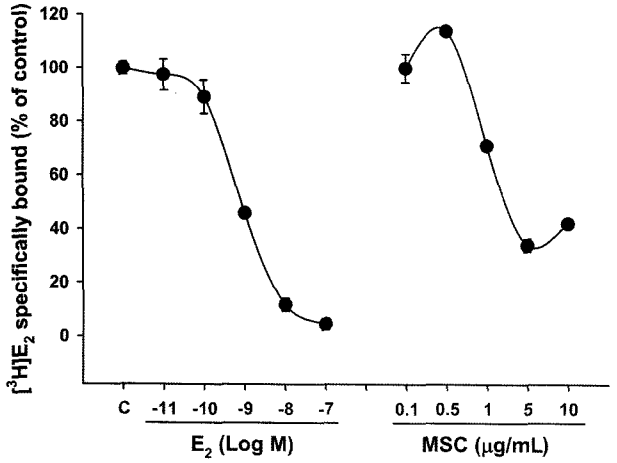

Fig. 3. Estrogen receptor binding affinity of MSC. The binding ability of 2.5 nM [2,4,6,7-^{3}H] E_2 to recombinant human ER in DMSO (C), E_2, and MSC was measured after the unbound radioligands were removed after incubation at room temperature for 4 h. The amount of the specific bound ligands was calculated by subtracting the nonspecific bound counts from the total. The results are expressed as the mean ± SD of three separate experiments.

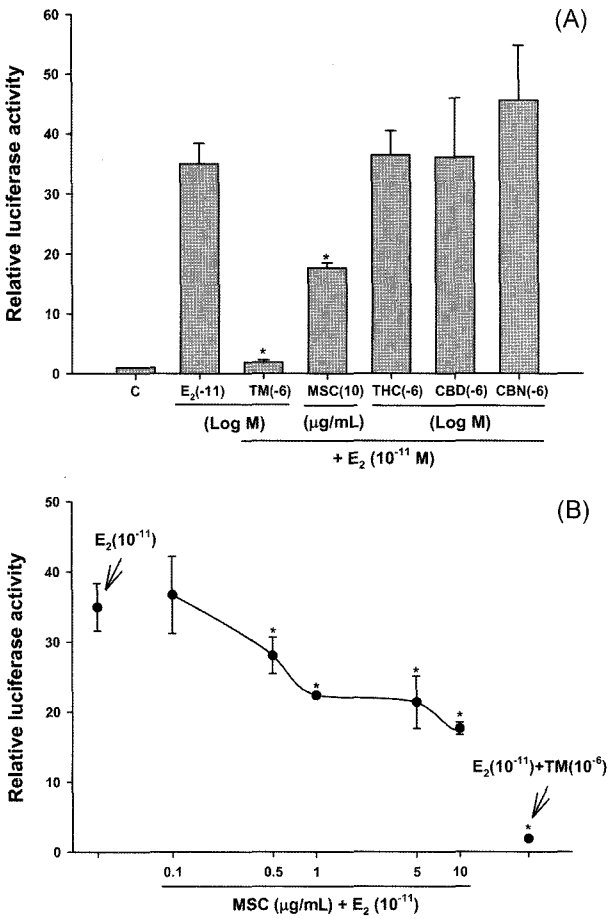

Fig. 4. Effects of MSC, THC, CBD, and CBN on the luciferase activity in MCF-7 cells measured using the reporter gene assay in the presence of E_2. (A) MCF-7 cells were treated with the indicated concentrations of E_2 or a combination of E_2 and TM or each compound for 24 h after being transiently transfected with the pERE2-luc construct using LipofectAMINE. DMSO was used as a control (C). The total luciferase activity was measured using a Luciferase assay reagent. The results are expressed as the mean ± SD of three separate experiments. Values significantly different from the control are indicated by an asterisk (*$p<0.05$ by Student's *t*-tests). (B) MCF7 cells were treated with the indicated concentrations of E_2 or a combination of E_2 and MSC at various concentrations (0.1-10 μg/mL) or TM (10^{-6} M) for 24 h.

determine if they can regulate the *in situ* and circulating estrogen levels. The CYP1A-dependent EROD activity is related to the stimulation of the metabolism of E_2. Therefore, this study evaluated the ability of these compounds to induce the EROD activity in H4IIE cells. As shown in Fig. 5, a dose-response curve was obtained for MSC only, which induced the highest level (39.9 ± 3.7% of TCDD) of the EROD activity at 5 μg/mL. Furthermore, THC, CBD, CBN, and MSC all stimulated the E_2 metabolism in a dose dependent manner, among which CBN significantly increased the E_2 metabolism by 51.8 ± 3.0% at a concentration of 10^{-6} M, compared with the effect of 10^{-9} M TCDD used as a positive control (Fig. 6).

Aromatase (CYP19) is a cytochrome P-450 that catalyzes the aromatization of androgens into estrogens and plays an important role in reproductive development as well as other biological responses. Fig. 7 shows the effects of the compounds on the aromatase activity in aromatase expressing human placental JEG-3 cells. MSC (0.01 – 10 μg/mL) inhibited the enzyme activity in a dose dependent manner, and 10 μg/mL MSC decreased the enzyme activity by more than 60% of that observed in the control (Fig. 7A, B). In contrast, 10^{-6} M THC, CBD, and CBN did not show any significant differences from the control (Fig. 7A).

Identification of biologically active fractions

The antiestrogenic effects of the fractionated samples of the MSC extract was further examined using the cell proliferation assay and the EROD assay in order to determine the causative chemical groups of MSC, which was demonstrated to be antiestrogenic. In the MCF7-BUS cell proliferation, F4 exhibited the strongest antiestrogenic activity, and 1-10 μg/mL F4 resulted in 72-84% of cell proliferation produced by E_2 (Fig. 8A, B).

The results of the EROD assay shows that F1, F2, and F4 produced an average 16 ± 0.5%, 12 ± 0.1%, and 21 ± 1.6% inductions of the enzyme activity, respectively. In addition, these fractions caused a concentration-dependent increase of the CYP1A enzyme activity. The remaining fractions did not have any significant activity (Fig. 9A, B). Therefore, this indicates that F4 is the most biologically active.

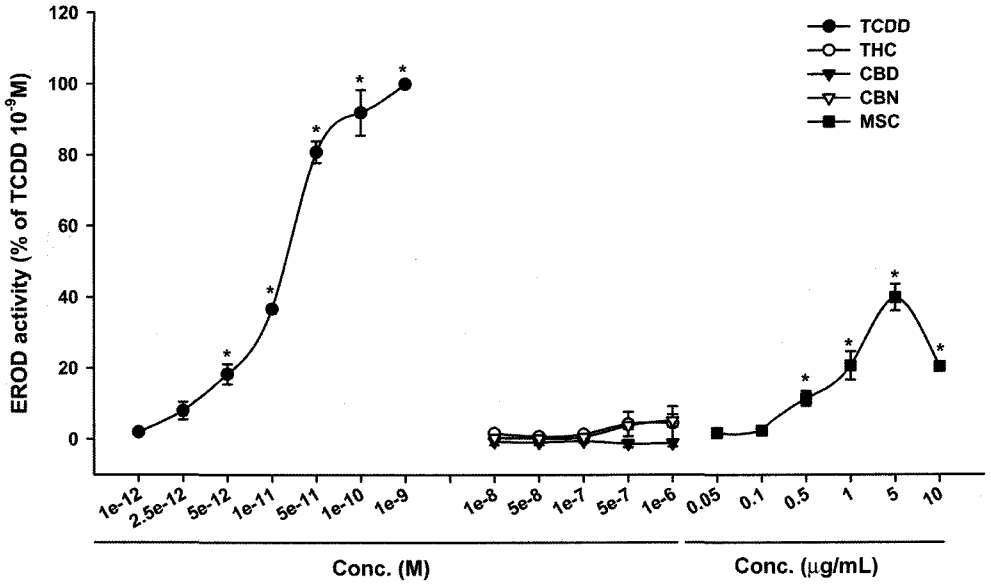

Fig. 5. Effects of THC, CBD, CBN, and MSC on the EROD activity in H4IIE cells. H4IIE cells were treated with TCDD (a positive control), THC, CBD, CBN, or MSC at various concentrations (10^{-12}-10^{-9} M for TCDD; 10^{-8}-10^{-6} M for THC, CBD, and CBN; 0.05-10 μg/mL for MSC) for 48 h. The EROD activity was measured as described in the Methods section. The results are expressed as the mean ± SD of three separate experiments. Values significantly different from the control are indicated by an asterisk (*$p<0.05$ by Student's *t*-tests).

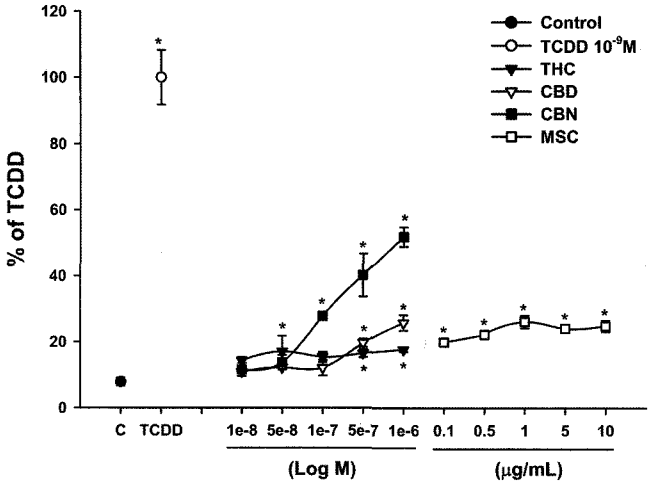

Fig. 6. Effects of THC, CBD, CBN, and MSC on the E_2 metabolism in MCF7-BUS cells. MCF7-BUS cells were treated with DMSO (a negative control), 10^{-9} M TCDD (a positive control), THC, CBD, CBN, or MSC at various concentrations (10^{-8}-10^{-6} M for THC, CBD, and CBN; 0.1-10 mg/mL for MSC) for 72 h. The effect on the E_2 metabolism was determined using the tritium release assay after treatment with 10 nM [^{3}H] E_2 for 24 h. The results are expressed as the mean ± SD of three separate experiments. Values significantly different from the control are indicated by an asterisk (*$p<0.05$ by Student's *t*-tests).

DISCUSSION

The incidence of marijuana smoking for recreational purposes has continuously increased on account of its easy availability. However, only a few studies have examined its harmful effects on the endocrine and reproductive systems. Marijuana smokers are exposed to not only marijuana plant elements, such as cannabinoids, but also combustion products. These pyrogenic chemicals, as well as cannabinoids, can disturb the steroid hormone system and some pyrogenic compounds from cigarette smoke condensate are antiestrogenic (Meek and Finch, 1999; Kamiya *et al.*, 2005). The estrogenic effects and ER binding abilities of cannbinoids and marijuana plant extracts have been examined (Rawitch *et al.*, 1977; Smith *et al.*, 1979; Sauer *et al.*, 1983; Ruh *et al.*, 1997; Watanabe *et al.*, 2005). However, these studies failed to explain the antiestrogenic effects of marijuana smoking. Therefore, this study examined the antiestrogenicity of MSC and cannabinoids along with the mechanism of their antiestrogenic action.

Antiestrogenic compounds can cause a biological response *via* a direct or indirect ER binding mechanism. In the classical ER-mediated pathway, the ligand binds to the ligand-binding domain of the ER followed by the formation of a liganded receptor homodimeric complex. The complex binds then to the distal EREs in the 5'-promoter regions of the E_2-responsive genes, further interacts with the components of the general transcription factor complex and coactivators or adaptors, and finally induces transcription. The antiestrogenic compounds act on the one hand by directly competing with E_2 for binding to the ER, resulting in a functionally inactive ligand bound complex (Korach *et al.*, 1997; Porter and Safe, 1999). On the other hand, they can force the metabolism of E_2 or restrain the biosynthesis of E_2, leading to the depletion of

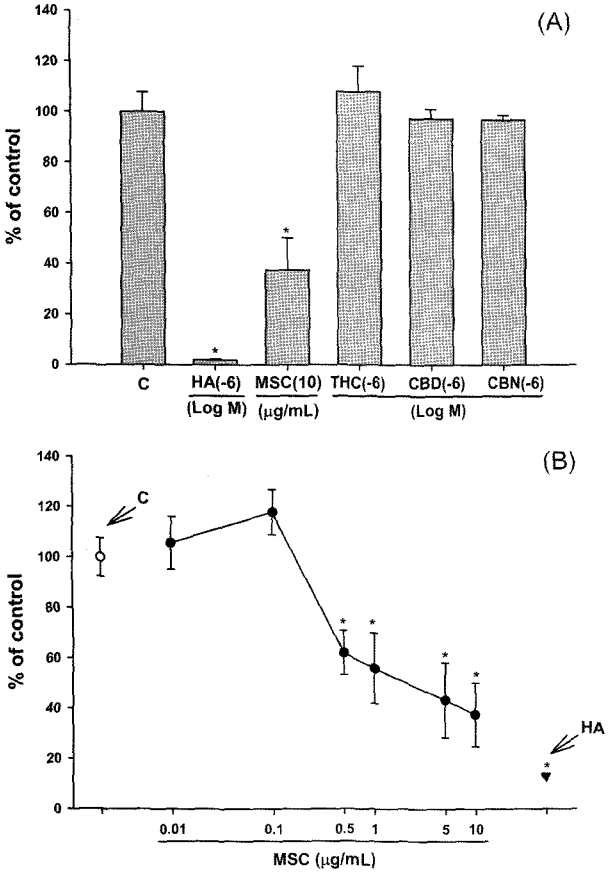

Fig. 7. Effects of MSC, THC, CBD, and CBN on the aromatase activity in JEG-3 cells. (A) JEG-3 cells were treated with the indicated concentrations of each compound for 24 h. DMSO (C) and 4-hydroxyandrostendione (HA) were used as a negative and positive control, respectively. The aromatase activity was determined using the tritium release assay after treating the cells with 54 nM [1β-^{3}H] androstenedione for 1 h. The results are expressed as the mean ± SD of three separate experiments. Values significantly different from the control are indicated by an asterisk (*$p<0.05$ by Student's *t*-tests). (B) JEG-3 cells were treated with various concentrations (0.1-10 μg/mL) of MSC for 24 h.

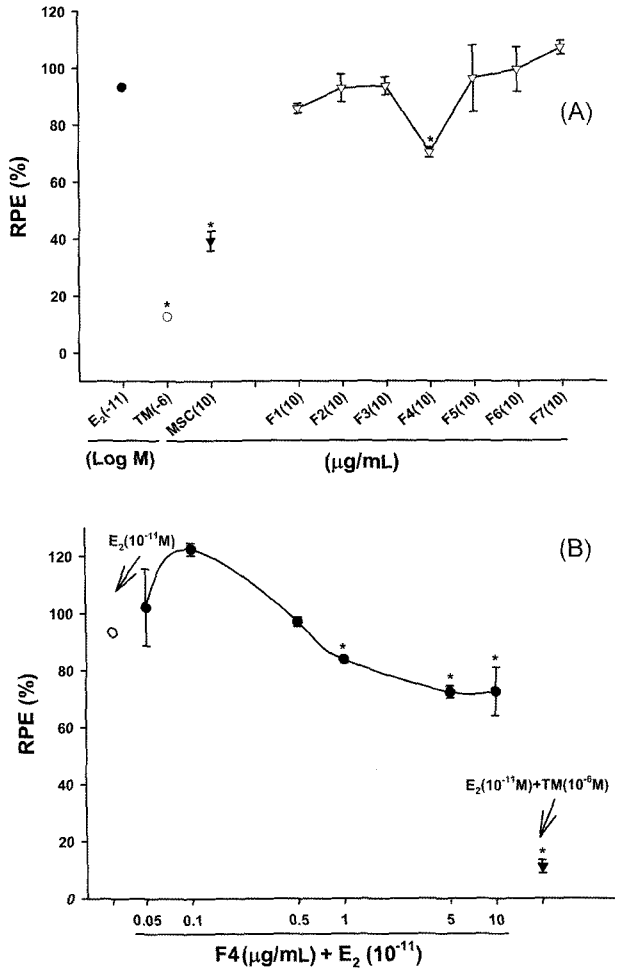

Fig. 8. Effects of seven fractionated samples on the MCF7-BUS cell proliferation in the presence of E_2. (A) MCF7-BUS cells were treated with the indicated concentrations of E_2 or combination of E_2 and TM, MSC, or each fraction (Fn, n=1-7) for 144 h. (B) MCF7-BUS cells were treated with the indicated concentrations of E_2 or a combination of E_2 and F4 at a range of concentrations (0.05-10 μg/mL) or TM (10^{-6} M) for 144 h. The assay was conducted in a similar manner to that reported in Fig. 2.

endogenous estrogen binding to the ER in an indirect manner. Previous studies reported that THC, CBD, and CBN did not competed with E_2 for the ER, and THC and CBD did not stimulate transcription of an EREtkCAT reporter gene transfected into MCF-7 cells (Sauer *et al.*, 1983; Ruh *et al.*, 1997; Watanabe *et al.*, 2005). Therefore, it was suggested that the cannabinoid compounds were not xenoestrogens. Marijuana plant extracts bound to the ER but the conclusions of the estrogenic activities were inconsistent (Sauer *et al.*, 1983; Watanabe *et al.*, 2005). Although MSC interacted with the ER (Sauer *et al.*, 1983), the (anti)estrogenic activity had not been demonstrated, and there have been no functional analyses of its mechanism of action in well-defined assays. Recent studies, demonstrating the presence of the antiestrogenic ingredients in cigarette smoke condensate (Meek and Finch, 1999; Kamiya *et al.*, 2005), have shown that MSC could have antiestrogenic potential. Therefore, this study investigated the antiestrogenic effects of MSC and cannabinoids related to ER-ligand binding as well as the regulation of endogenous estrogen. MSC was further fractionated by liquid-liquid partitioning, which was followed by silica gel column chromatography, and the fractions were traced by the cell proliferation assay to identify the causative agents related to the ER-mediated pathway.

In this study, the antiestrogenic effects of MSC and the cannabinoid compounds through ER-ligand binding were examined using the ER-positive human mammary carcinoma cell lines, MCF7-BUS and MCF-7 cells. In the

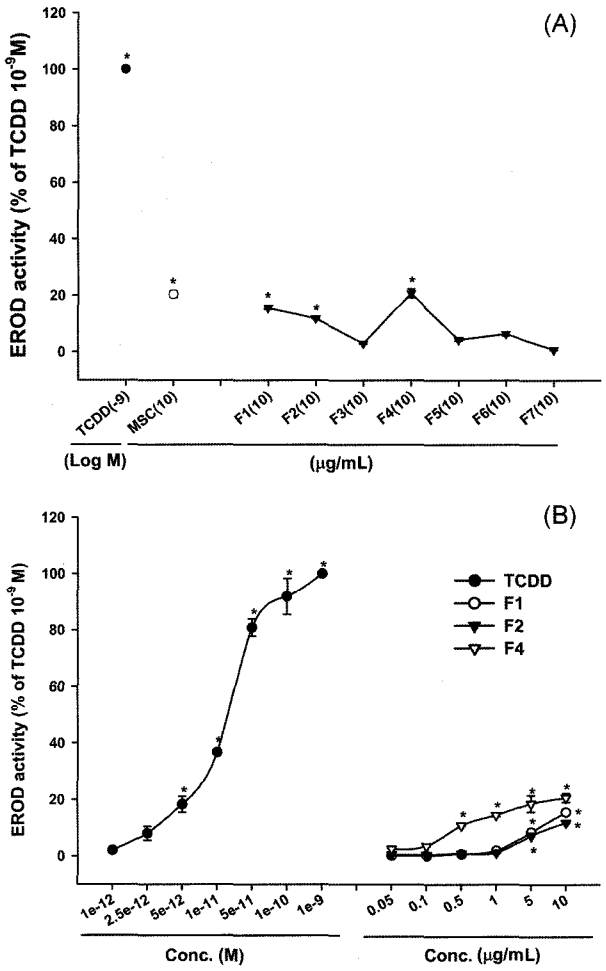

Fig. 9. Effects of seven fractionated samples on the EROD activity in H4IIE cells. (A) H4IIE cells were treated with the indicated concentrations of TCDD, MSC, or each fraction (Fn, n=1-7) for 48 h. (B) H4IIE cells were treated with TCDD, F1, F2, or F4 at a range of concentrations (10^{-12}-10^{-9} M for TCDD; 0.05-10 μg/mL F1, F2, and for F4) for 48 h. The assay was carried out in a similar manner to that reported in Fig. 5.

cell proliferation and reporter gene assay, only MSC had both ER- and ERE-mediated antiestrogenic effects in a dose-dependent manner, while THC, CBD, and CBN did not. The affinity of MSC for the ER was confirmed using the competitive ER binding assay. The results clearly demonstrated that the antiestrogenicity of marijuana was induced *via* the typical ER-mediated pathway by binding the ER-MSC complex to ERE. Although cannabinoids have similar carbocyclic structures to steroids (Martin, 1986), they did not interfere with [^{3}H] E_2 binding to the ER. Therefore, it is unlikely that physiological antiestrogenic effects on marijuana abusers are due to cannabinoids.

It was reported that marijuana and cannabinoids affect the female reproductive function by altering multiple hormonal systems, including prolactin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estrogen. In females, marijuana administration suppresses estrogen controlled by the hypothalamic-pituitary-gonadal axis, where FSH and LH act on the gonads producing steroid hormones. However, animal studies with THC have reported inconsistent results (Brown and Dobs, 2002; Rosenkrantz *et al.*, 1980). The estrogen level is a critical factor in the human physiology in that it influences the development, sexual differentiation, fertility, control of the female reproductive tract organ responsiveness, and female diseases due to hormone imbalances (Korach *et al.*, 1997).

This study showed that MSC enhanced the EROD activity in a dose dependent manner, but THC, CBN, and CBD did not. Roth *et al.* (2001) demonstrated that marijuana tar induced the EROD activity and THC activated transcription of CYP1A while acting as a competitive inhibitor of CYP1A in the EROD assay. They explained that the tar stimulated CYP1A because marijuana cigarettes produced PAHs during burning. They also suggested that THC could act as an antagonist because cannabinoids were another class of cyclic aromatic hydrocarbons that interact with the AhR. The result of another EROD assay with the fractionated samples aimed at identifying the possible group of compounds responsible for the enhanced EROD activity of MSC showed that F1, F2, and F4 significantly stimulated the EROD activity. Generally, according to polarity fractionation, chemicals that can be detected in each fraction are as follows (Topinka *et al.*, 1998): organic bases (F1, nitrated heterocyclic compounds like pyridins, chinolins, and acridins); organic acids (F2, aliphatic acids, anhydrides of aromatic acids, alcohols, phenols, and aldehydes); aliphatic fraction (F3, aliphatic and chlorinated hydrocarbons); aromatic fraction (F4, PAHs and their alkylderivatives); slightly polar fraction (F5, nitro-PAH, ketones, and quinones); moderately polar fraction (F6, phthalates and oxyderivatives of PAH); highly polar fraction (F7, alcohols and esters of carboxylic acids). It was reported that marijuana produces more than 150 PAHs, some of which are both ER antagonists and AhR agonists (Nahas and Latour, 1992). Therefore, F4 might induce the EROD activity *via* the action of PAHs. In addition, CYP1A induction by pyridines and heterocyclic aromatic amines among the ingredients presented in F1, which are major constituents of cigarette smoke, was demonstrated (Iba *et al.*, 2002; Hummerich *et al.*, 2004; Smith *et al.*, 2004). There are conflicting interpretations for the increased EROD activity in F2. Alkylphenols, a group of pyrogenic components presented in F2, inhibited the CYP1A activity (Hasselberg *et al.*, 2004) but some plant phenolic compounds, which are another group of possible components in F2, upregulated CYP1A (Szaefer *et al.*, 2003; Gross-Steinmeyer *et al.*, 2004). Since a fractionated sample is still a complicated mixture, further examinations will be needed to determine the EROD activity of F2.

Some studies have reported that the CYP1A-dependent EROD activity can stimulate the E_2 metabolism to the weak estrogen, 2-hydroxyestrone (2-OHE1) (Schneider *et al.*, 1984; Dannan *et al.*, 1986; Spink *et al.*, 1992; Suchar *et al.*, 1995; Bradlow *et al.*, 1996; Arcaro *et al.*, 1999) and inhibit the aromatase activity (Drenth *et al.*, 1998). Therefore, this study investigated the effects of MSC and the related cannabinoids on the ability of estrogen secretion by the metabolism and biosynthesis of E_2. MSC disturbed the biosynthesis of estrogen by inhibiting the aromatase activity. THC, CBD, and CBN stimulated the E_2 metabolism, among which CBN produced a twofold induction of the metabolism more than MSC. However they did not block the biosynthesis of estrogen. In humans, CYP3A and 1B as well as CYP1A lead to the 2-, 4-, or 16α-hydroxylation of E_2 or estrone in the metabolic pathway (Shou *et al.*, 1997; Yamazaki *et al.*, 1998; Tsuchiya *et al.*, 2005). Therefore, it was presumed that the E_2 metabolism caused by THC, CBD, and CBN might be the result of other metabolizing enzymes, such as CYP 3A and 1B, which were not detected by the EROD assay. It is possible that not only other complex compounds in MSC but also cannabinoids can induce the antiestrogenic responses *in vivo* by depleting the E_2 level.

The MCF7-BUS cell proliferation assay was carried out on the fractionated samples in an attempt to determine the causative group for the antiestrogenic activity. It was found that the non-polar fraction (F4) of MSC had the strongest biological activity among the seven fractions. This corresponds to reports showing that there are aromatic compounds including PAHs present in F4 (Topinka *et al.*, 1998), and PAHs elicit the antiestrogenic effects that are mediated *via* cross-talk between the ER and the AhR (Chaloupka *et al.*, 1992; Safe *et al.*, 2003). Therefore, although this fraction is still extremely complex mixture, it is assumed that PAHs are one of the causative chemical groups for the antiestrogenic activities of marijuana smoke.

In conclusion, the antiestrogenic effect of MSC is mediated by the ER. In addition, the ER-AhR cross-talk might be related to the antiestroginicity as a result of the enhancement of the E_2 metabolism and the depletion of the intracellular hormone. These findings suggest that pyrogenic products including PAHs in the non-polar fraction are responsible for the antiestrogenic effect of MSC. A further study will be needed to identify the chemical character of the non-polar fraction using chemical analysis.

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