Huaier aqueous extract suppresses human breast cancer cell proliferation through inhibition of estrogen receptor α signaling

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Abstract. Estrogen receptor α (ERα) has been reported to play a critical role in promoting the growth of breast tumor cells. In the present study, we explored the effect of Huaier extract on estrogen receptor α signaling in breast cancer cell lines. Our data demonstrated that Huaier extract effectively inhibited the proliferation of the MCF-7, T47D and ZR-75-1 human breast cancer cell lines. For the mechanism analysis, we demonstrated that Huaier extract significantly reduced the mRNA and protein levels of ERα in all three ERα-positive cell lines. The downregulation of ERα protein levels was correlated with activation of the proteasomes. We demonstrated that Huaier extract markedly decreased the expression of both ERα and its downstream genes, inhibited the estrogen-stimulated proliferation and reversed the estrogen-induced activation of the nuclear factor κB (NFκB) pathway. Our study provides evidence that Huaier extract is a novel estrogen receptor modulator and is a promising drug for the prevention and treatment of ERα-positive human breast cancers.

Introduction

Estrogen receptors (ERα and ERβ) are members of the superfamily of nuclear steroid hormone receptors (1). After the binding of hormone to these receptors, the hormone-receptor complexes directly bind to specific sequences on the DNA, named estrogen response elements (EREs), or interact with other transcription factors (2-4). ERα and ERβ are expressed both in normal and cancerous breast epithelium (5). It has been reported that a high ERα:ERβ ratio was responsible for enhanced cell proliferation (6). Consistent with the critical role of ERα in mediating the proliferative effect of estrogen, mice that selectively lacked ERα showed a severely under-developed mammary epithelium with only rudimentary ductal structure (7,8). In 2003, Shaaban *et al* demonstrated that the malignant progression of breast lesion was correlated with elevated ERα expression (9). Therefore, selective disruption of ERα signaling in breast cancer cells may be a potent therapy to prevent the development of breast cancers.

Current selective estrogen receptor modulators (SERMs) exhibit their effects through three different ways (10): i) interfering with estrogen receptor α (ERα) binding, such as tamoxifen and faslodex; ii) inhibiting endogenous estrogen production, such as oophorectomy, anastrozole and letrozole; and iii) downregulating ERα protein level, such as fulvestrant. SERMs may exert estrogen agonist action in some target tissues while acting as estrogen agonist action in others (11). Therapeutical SERMs are considered to be able to inhibit the proliferation of breast cancer without the influence on bone density, cholesterol level or uterine endometrium (12,13).

Natural products have long been considered as a rich source of novel bioactive chemicals providing potential effective new drugs. Epidemiological and physiological studies have shown that phytochemicals from vegetables and fruits possess potent anticancer activities with minimal to no side-effect (14). Huair extract is a kind of officinal fungi and has been used in China for nearly 1,600 years. In the past few years, its anticancer activities are attracting increasing interest. Our previous studies showed that the antitumor effect of Huaier extract on breast cancer may be associated with induction of apoptosis and inhibition of angiogenesis (15,16). Similar effects were seen in human liver cancer (17-19). In view of these findings, it was of great interest to determine whether Huaier extract could affect the ER signaling pathway in human breast cancer cells.

In this study, we used three ERα-positive breast cancer cell lines (MCF-7, T47D and ZR-75-1). Our findings demonstrated that Huaier extract markedly decreased expression of both ERα and its downstream genes, inhibited the estrogen-stimulated proliferation and reversed the estrogen-induced activation of the NF-κB pathway. These effects provided a further rationale for the use of Huaier extract in the prevention and treatment of ERα-dependent human breast cancers.

Materials and methods

Cell cultures and reagents. Human breast cancer cell lines, MCF-7, T47D and ZR-75-1, were purchased from ATCC (Manassas, VA, USA). MCF-7 cells were routinely maintained in DMEM medium (Gibco-BRL, Rockville, IN, USA) supplemented with 10% fetal bovine serum (Haoyang Biological Manufacture Co. Ltd., Tianjin, China), 100 U/ml penicillin and 100 μg/ml streptomycin. T47D cells were cultured in RPMI-1640 medium

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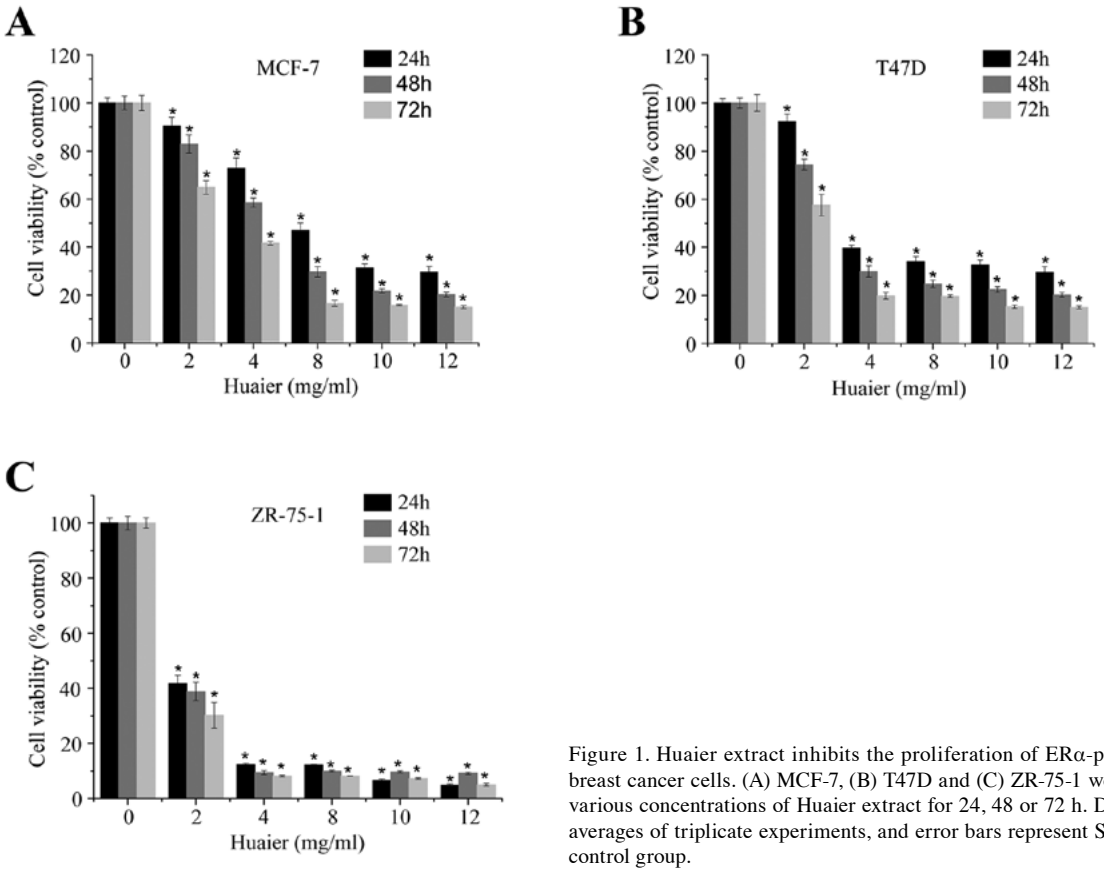

Figure 1. Huaier extract inhibits the proliferation of ERα-positive human breast cancer cells. (A) MCF-7, (B) T47D and (C) ZR-75-1 were exposed to various concentrations of Huaier extract for 24, 48 or 72 h. Data shown are averages of triplicate experiments, and error bars represent SD. *P<0.05 vs. control group.

(Gibco-BRL) with 10% fetal bovine serum and 10 μg/ml bovine insulin (Sigma-Aldrich, St. Louis, MO, USA). ZR-75-1 cells were cultured in the above medium in the absence of bovine insulin. For estrogen-free experiments, medium was changed to phenol red-free DMEM or RPMI-1640 with 0.5% dextran-coated charcoal (DCC) stripped FBS (Haoyang Biological Manufacture) for 72 h. The medium was then changed to the indicated serum and phenol red-free medium for 24 h before various treatments as described in the figure legends (20,21). Electuary ointment of Huaier extract was kindly provided by Gaitianli Medicine Co. Ltd. (Jiangsu, China). The stock solutions were generated by dissolving electuary ointment in indicated medium (15,16). MG 132 was purchased from Sigma-Aldrich. β-estradiol (E2) was obtained from Wako and was dissolved in ethanol with a final concentration of ethanol in medium of less than 0.1%. Anti-ERα antibody was from Dako. All other antibodies were purchased from Cell Signaling Technology.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated into 96-well plates and grown overnight at 37˚C in 5% CO_2 for 24 h. After treatment with the appropriate concentration of Huaier extract for indicated time, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated for 4-6 h at 37˚C. The formazan crystal was dissolved in DMSO for 10 min. Optical density (OD) was measured by the Microplate Reader (Bio-Rad, Hercules, CA, USA).

For E2-stimulated proliferation, cells were seeded in 96-well plates in phenol red-free medium in the presence of 10% charcoal-stripped fetal bovine serum. The next day, the medium was changed to fresh serum and phenol red-free medium for another 24 h. Then 10 nM E2 in the presence or absence of different concentrations of Huaier extract was added for 48 h.

Western blot analysis. For western blot analysis, treated cells were washed with ice-cold PBS and lysed in a modified RIPA buffer (1X PBS, 1% NP-40, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 0.5% sodium deoxycholate and 1 mM sodium orthovanadate) with protease inhibitors for 20 min at 4˚C. After centrifugation at 12,000 rpm for 15 min at 4˚C, the supernatant was collected. Subsequently, 50 μg of total cellular protein from each sample were separated by 10% SDS-PAGE and electrotransferred onto a polyvinylidene fluoride (PVDF) membranes by a semi-dry blotting apparatus (Bio-Rad). Then the membranes were immunoblotted with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (1:7,000). Labeled protein spots were visualized by ECL (PerkinElmer) according to the manufacturer's instruction. β-actin was used as the loading control.

Quantitative real-time reverse transcriptase (RT)-PCR. Total RNA was isolated from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and methods described previously (22). Briefly, total RNA from treated cells were isolated with TRIzol reagent according to the manufacturer's protocol. Total RNA was used to synthesize cDNA using PrimerScript RT Reagent kit (Takara). The cDNA reaction product was amplified with following primers: ERα forward, 5'-CGGCATTCTACAGGCCAAATT-3' and ERα reverse, 5'-AGCGAGTCTCCTTGGCAGATT-3'; PR forward, 5'-AGCC

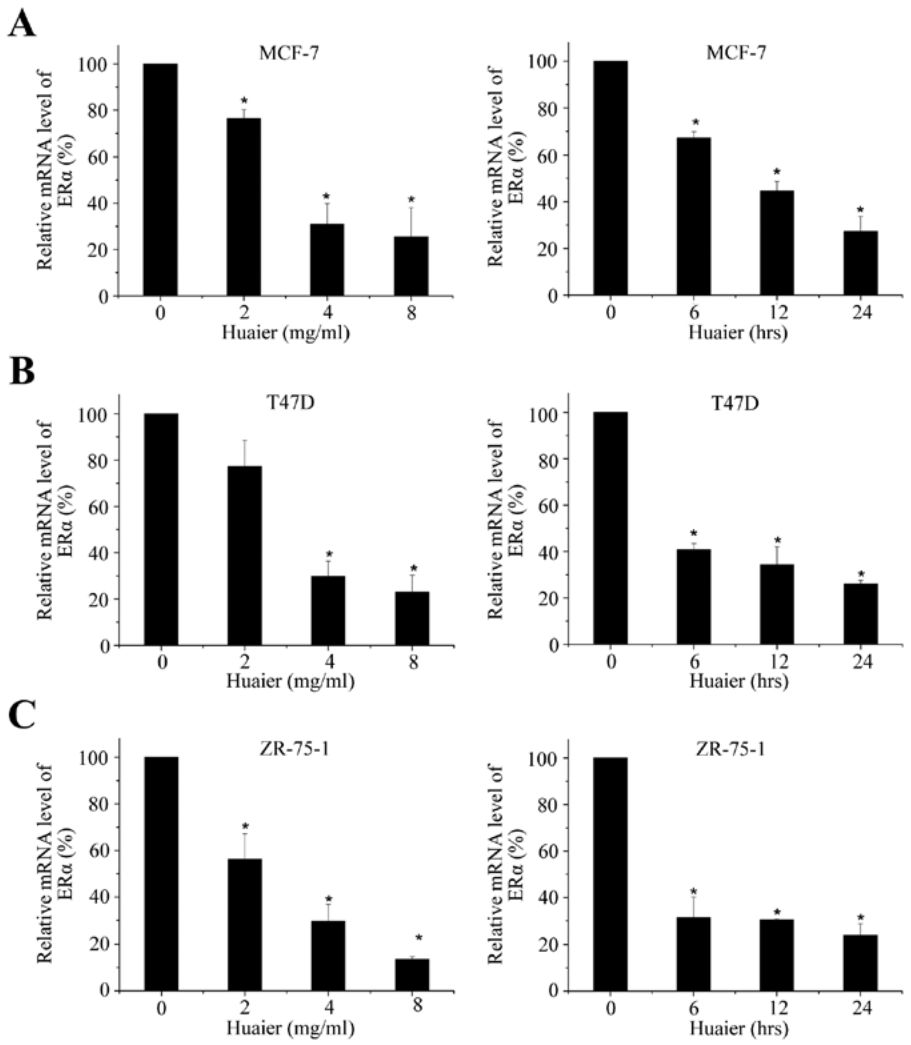

Figure 2. Huaier extract significantly decreases the mRNA levels of ERα in all three ERα-positive human breast cancer cell lines. (A) MCF-7, (B) T47D and (C) ZR-75-1 cells were treated with various concentrations of Huaier extract for indicated time. The mRNA levels were detected through real-time PCR. GAPDH was used as internal control for normalization. Data are representative of three independent experiments. *P<0.05.

CACAATACAGCTTCGAG-3' and PR reverse, 5'-TTTCGAC CTCCAAGGACCAT-3'; pS2 forward, 5'-TGACTCGGGGTC GCCTTTGGAG-3' and pS2 reverse, 5'-GTGAGCCGAGGC ACAGCTGCAG-3'; cathepsin D forward, 5'-TGAGGCCATT GTGGACAAGGCAC-3' and cathepsin D reverse, 5'-GTCACG GTCAAACACAGTGTAGTAG-3'; GAPDH forward, 5'-AGAA GGCTGGGGCTCATTTG-3' and GAPDH reverse, 5'-AGGG GCCATCCACAGTCTTC-3'. GAPDH was used for equal RNA loading. The experiments were repeated for at least three times.

Statistical analysis. Data were expressed as means ± standard deviations (SD) for three replicate experiments and considered significant at P<0.05. The statistical analysis was carried out by using SSPS edition 19.0.

Results

Huaier extract effectively inhibited the proliferation of ERα-positive breast cancer cell lines. We first examined the effect of Huaier extract on the growth of three ERα-positive human breast cancer cell lines, MCF-7, T47D and ZR-75-1. As shown in Fig. 1, Huaier extract significantly inhibited the proliferation of these three cell lines in a dose- and time-dependent manner (P<0.05). The results from MCF-7 were similar to our previous reports (15). In addition, at 24 h, the respective IC_{50} values for MCF-7 (Fig. 1A), T47D (Fig. 1B) and ZR-75-1 (Fig. 1C) were 7.27±0.86, 5.12±0.71 and 1.30±0.11 mg/ml. Thus the ZR-75-1 cell line was more sensitive to the growth inhibition caused by Huaier extract than the other two.

Huaier extract effectively decreased the mRNA and protein levels of ERα in three ERα-positive human breast cancer cell lines. ERα is generally associated with cell cycle progression and has been reported to promote both anchorage-dependent and anchorage-independent growth of breast cancer cells (23-25). To determine the effect of Huaier extract on the level of ERα, cell lines were exposed to various concentrations of Huaier extract for 24, 48 or 72 h. We then examined the effect of Huaier extract on ERα mRNA levels. As shown in Fig. 2A, Huaier extract could decreased the mRNA level of ERα dose- and time-dependently in MCF-7 cell line. After various times of 4 mg/ml Huaier treatment, the transcriptional levels were significantly inhibited

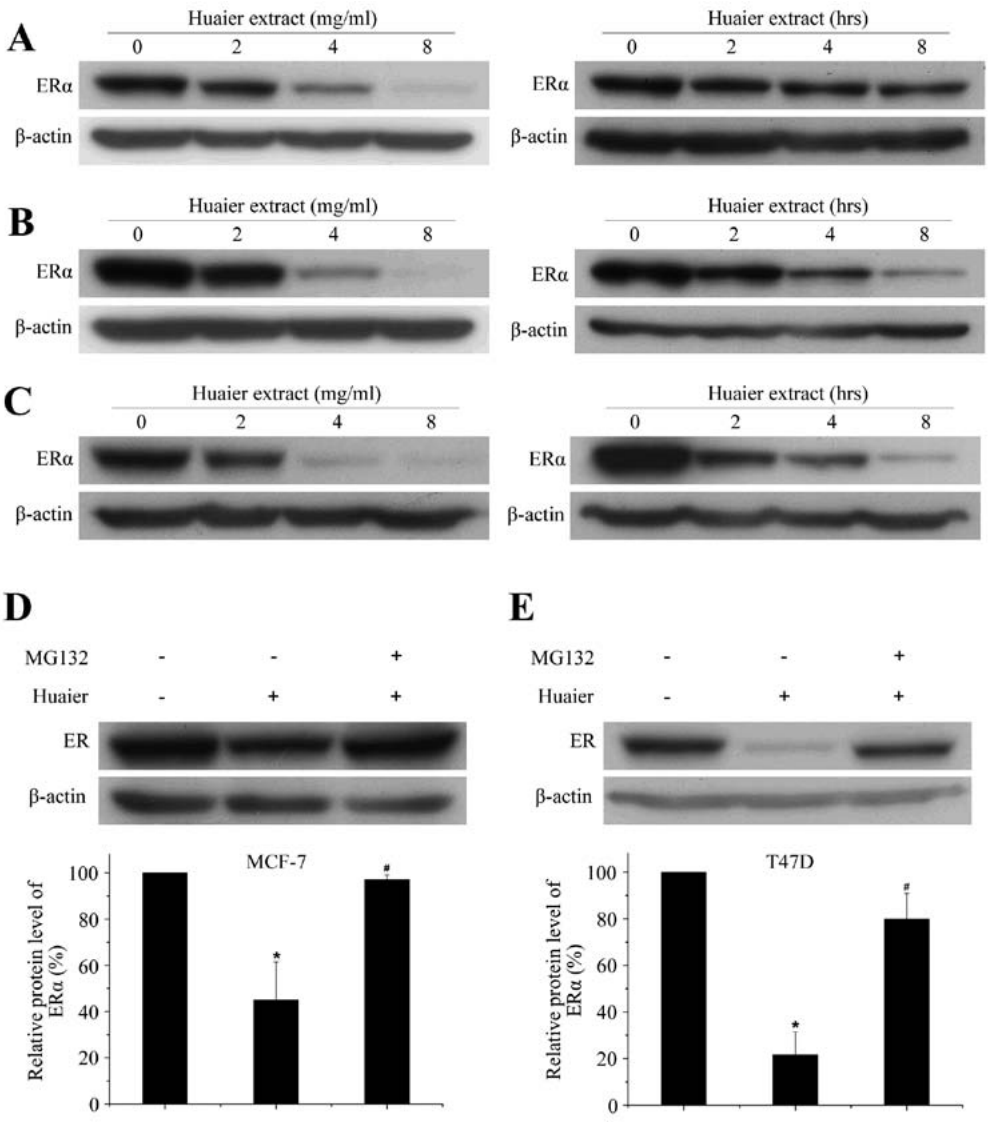

Figure 3. Huaier extract significantly reduces the protein levels of ERα through activated proteasome pathway. ERα levels were examined by western blot assay. Actin was used as a loading control. (A-C) After treatment with Huaier extract, ERα levels in MCF-7, T47D and ZR-75-1 were reduced in a dose- and time-dependent manner. (D and E) MCF-7 and T47D cells were treated with vehicle, 4 mg/ml Huaier extract or 10 μm MG 132 in combination with Huaier extract for 24 h. MG 132 was added 1 h before the Huaier extract treatment. The bar graphs represent the mean relative densities ± SD of three independent experiments. *P<0.05 with respect to the basal conditions. #P<0.05 with respect to Huaier treatment alone.

by 32.9±2.7, 55.4±4.1 and 72.7±6.3% (P<0.05), respectively. Reduction of ERα mRNA levels was also observed in T47D and ZR-75-1 cells (Fig. 2B and C).

Data from Fig. 3 demonstrated that Huaier extract markedly downregulated the ERα protein levels in a time- and dose-dependent manner (P<0.05). After 24 h incubation of 4 mg/ml Huaier extract, the levels of ERα were reduced by 67.2±9.8, 86.1±7.0 and 85.3±4.0% in MCF-7 (Fig. 3A), T47D (Fig. 3B) and ZR-75-1 (Fig. 3C) cell lines, respectively. Reduction of ERα protein levels was observed in all three ERα-positive breast cancer cell lines, suggesting that the impact of Huaier extract on this protein is common to ERα-positive breast cancer cell lines.

Huaier-induced downregulation of ERα protein was associated with activated proteasome. Previous studies have revealed that regulation of ERα protein is associated with protein degradation via proteasomes (26). To explore the involvement of proteasome in the Huaier-induced ERα degradation, the proteasome inhibitor, MG132, was used. Treatment of MCF-7 cells with Huaier extract led to a potent and persistent decrease in ERα protein levels. Addition of MG132 before Huaier treatment markedly rescued the downregulation of ERα (P<0.05, Fig. 3D). In addition, MG132 was also able to abolish the inhibitory effect of Huaier on ERα protein in T47D cell line (Fig. 3E). These findings demonstrated that inhibition of both gene transcription and proteasome-mediated degradation was required to reduce endogenous levels of ERα in response to Huaier extract treatment.

Huaier extract significantly downregulates the transcription of estrogen responsive genes. It has been reported that some activators of ERα, such as 17-β-estradiol, could also decrease ERα level (27), suggesting that a reduced level of ERα was not

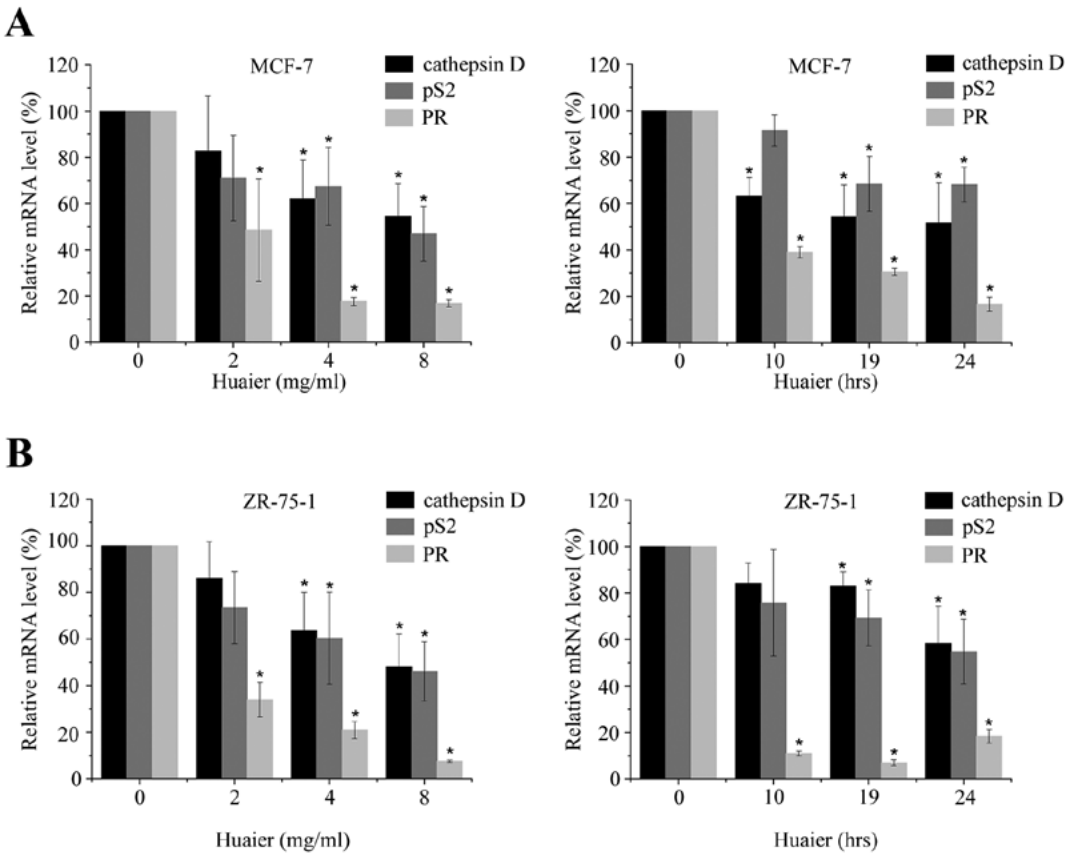

Figure 4. Huaier extract reduces the levels of target genes regulated by estrogen receptor. (A) MCF-7 and (B) ZR-75-1 cells were treated with various concentrations of Huaier extract for 10, 19 or 24 h. mRNA levels of cathepsin D, pS2 and PR were determined by real-time PCR as described in Materials and methods. Data are representative of three independent experiments. *P<0.05.

closely associated with a reduction of ERα activity. Therefore, we next determined the transcriptional activity of ERα by detecting its downstream target genes after Huaier extract treatment (28,29).

The expressions of cathepsin D, pS2 and progestogen receptor (PR) were reported to be regulated by estrogen (30). To further determine the transcriptional activity of ERα, we examined their mRNA levels after Huaier extract treatment within 24 h. The levels of transcription of each gene were normalized to the level of GAPDH. As shown in Fig. 4A, MCF-7 cells were cultured in the absence or presence of Huaier extract (2-8 mg/ml) for 10, 19 or 24 h. Significant reduction of mRNA could be observed dose- and time-dependently (P<0.05). Similar results were obtained in ZR-75-1 cells (Fig. 4B). However, as PR expression in T47D cells was demonstrated to be independent of estrogen (31), we did not detect the mRNA level of PR in T47D cells. In addition, Huaier extract failed to cause significant reduction on gene expression of pS2 and cathepsin D in T47D cells (data not shown).

Huaier extract inhibits E2-induced proliferation and NF-κB activation. The proliferation of ERα-positive human breast cancer cell lines is strongly stimulated by estrogens (25). To specifically determine the inhibitory effect of Huaier extract on E2-induced effect, MTT and western blot assays were used. As shown in Fig. 5A, Huaier extract significantly inhibited E2-induced cell proliferation in a dose-dependent manner. The survival of MCF-7 cells was significantly reduced by 49.1±4.6% after 48-h treatment with 1 mg/ml Huaier extact as compared with E2 treatment alone. The inhibition was observed in both MCF-7 and T47D cell lines (Fig. 5A and B).

It has been reported that activation of NF-κB contributed to the estrogen-induced proliferation in breast cancer cells (32). Thus, we detected the effect of Huaier extract on NF-κB pathway. The activation of NF-κB was assessed at 1 h in MCF-7 and T47D cells treated with control, estrogen alone or in combination with Huaier extract (2-4 mg/ml). As shown in Fig. 5C and D, 10 nM estrogen significantly phosphorylated P65 without influencing the total protein level of P65. After addition of Huaier extract, the phosphorylated levels of P65 were reduced almost to the basal level without estrogen. These results demonstrated that Huaier extract completely abolished the effect of estrogen on the activation of NF-κB and thus suppressed the proliferation of breast cancer cells induced by estrogen.

Discussion

ERα has become an important target in the treatment of hormone-responsive breast cancers. It has been reported that treatment with tamoxifen has enhanced patient survival (33). Unfortunately, most patients initially responding to anti-estrogen therapies, such as tamoxifen, will eventually

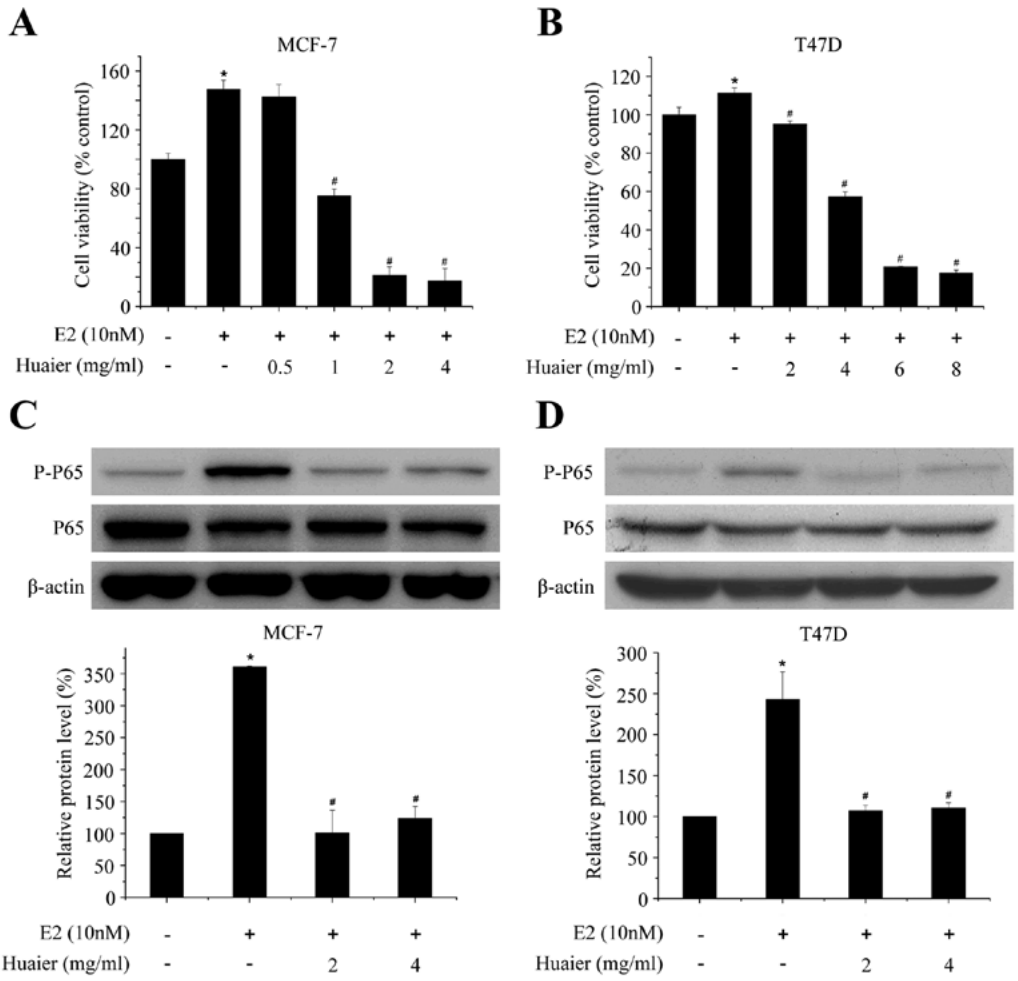

Figure 5. Huaier extract inhibits the cell growth and activation of NF-κB pathway stimulated by estrogen. (A and C) MCF-7 and (B and D) T47D cells were maintained in the serum- and phenol red-free medium in the absence or presence of 10 μM E2 alone or in combination with differen t concentrations of Huaier extract. (A and B) Cell viability was determined using MTT assay after incubation of the above medium for 48 h. (C and D) Western blot assay was performed after presence of the above medium for 1 h. The results were obtained from three independent experiments and are shown as the mean ± SD. *P<0.05 with respect to the basal conditions. #P<0.05 with respect to E2 treatment alone.

become resistant (34), along with a possible association with endometrial carcinomas (35,36). Although the mechanisms of endocrine resistance are not fully identified, cross-talk between ERα and growth factor signaling pathways may be involved (37,38). Therefore, downregulating the levels of ERα may be a potent therapeutic therapy for both primary estrogen-dependent breast cancer and hormone-refractory breast cancer.

Fulvestrant (ICI 182780) is a selective estrogen receptor downregulator (SERD) with no agonist effects and has been demonstrated to decrease the level of ERα protein to block both ligand-dependent and -independent receptor activation (39). However, recent studies have reported that fulvestrant could cause gastro-intestinal disturbance and hot flashes (40). Thus, alternative interventions such as compounds from natural products are needed to replace or to supplement current therapies.

Recently, Huaier extract was shown to exhibit antitumor and anti-angiogenesis activities both *in vitro* and *in vivo* (15,16). Studies in our laboratory have demonstrated that Huaier extract inhibited breast cancer growth via a direct pro-apoptotic effect on tumor cells, as well as through an indirect effect on endothelial cells (15,16). However, little is known about the effect of Huaier extract on the estrogen receptor signaling pathway in breast cancers.

In the present study, we explored the mechanisms of action of Huaier extract on breast cancer cell growth by focusing on ERα. Following the treatment with various concentrations of Huaier extract, the mRNA and protein levels of ERα were significantly decreased in MCF-7, ZR-75-1 and T47D cell lines in a time- and dose-dependent manner (Figs. 2 and 3), suggesting that this effect was general in ERα-positive breast cancer cells. In search for detail mechanisms causing reduced ERα level, we observed that MG 132, a proteasome inhibitor, could effectively suppress Huaier-induced ERα degradation (Fig. 3D and E). Thus, our data demonstrated that Huaier extract reduced the mRNA levels of ERα and decreased its protein through promotion of the proteasome pathway. However, fulvestrant reduce ERα levels only through increasing protein turnover without affecting its mRNA levels (39). In this view, Huaier extract may be useful in the treatment of breast cancer that are resistant to the pure antiestrogen.

After binding to ERα, the estrogen-ERα complex will translocate to the target DNA binding site, the estrogen responsive element, in the promoter region of the target gene for gene transcription activation (41). Therefore, we next examined the expression level of downstream genes regulated by estrogen, such as cathepsin D, pS2 and PR (30). After treatment by Huaier extract, the mRNA level of these genes were significantly reduced in a time- and dose-dependent manner (Fig. 4), suggesting that the Huaier extract suppressed the transcription activity of ERα along with its reduced levels.

To further examine the effect of Huaier extract on the 17β-estradiol-stimulated cell growth and its potential mechanisms, we analyzed several pathways regulated by estrogen and discovered NF-κB pathway. Although some studies showed that estrogen inhibited the tumor necrosis factor-α-induced activation of NF-κB (42), Rubio *et al* demonstrated no antagonism between ER and NF-κB in T47D and HC11 cells (32). In addition, in their study, activation of NF-κB was needed for estrogen-induced proliferation and expression of cyclin D1. In our study, data from Fig. 5 shows that 17β-estradiol significantly enhanced the phosphorylation of P65, the NF-κB component, in a phenol red and serum-free condition. After addition of Huaier extract, the level of P-P65 was reduced to the basal level without estrogen. Therefore, we suggest that Huaier extract inhibited the E2-induced proliferation of breast cancer through activation of the NF-κB pathway.

In summary, we found that Huaier extract potently inhibited the proliferation of ERα-positive breast cancer cells, and identified ERα as a possible target for Huaier extract treatment. Therefore, it is of great importance to develop this natural product to treat or prevent ERα-positive breast cancers.

Acknowledgements

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