Quercetin Induces Oxidative Stress and Potentiates the Apoptotic Action of 2-Methoxyestradiol in Human Hepatoma Cells

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Hepatocellular carcinoma (HCC) is the leading cause of cancer mortality in Asia. This study evaluated the growth inhibition effect of quercetin and 2-methoxyestradiol in vitro in human HCC cell lines. Combination treatment enhanced the cytotoxic effect in HA22T/VGH and HepG2 cell lines as compared with quercetin or 2-methoxyestradiol alone. The cell population of sub-G0/G1 phase and the level of annexin V binding were increased synergistically after combination treatment with quercetin and 2-methoxyestradiol in both cell lines. Moreover, quercetin combined with 2-methoxyestradiol increased superoxide levels, mitochondrial superoxide dismutase (MnSOD) in mRNA, protein levels, and SOD activity. Finally, we also found the mitochondrial potential was decreased after combination treatment. The changes of reactive oxygen species and mitochondrial disruption were likely to be involved in the mechanism for the synergistic cytotoxicity effects of combination treatment in human hepatoma cells. These results provided a basis for further study of the potential usage of quercetin combination with hormonal agents for the treatment of human hepatoma.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and is also the leading cause of death in cancer patients in Taiwan (1). Currently, total removal of tumor by surgery is the most effective treatment for HCC. However, surgical treatment has limitations for patients with large or multiple tumors. Moreover, after tumor resection, the recurrence rate of HCC was quite high (2). Therefore, it is not only important but also necessary to search for effective anticancer agents to improve the survival rate of patients with advanced or recurrent HCC after surgical treatment. Since high-dose treatment with anticancer drugs may induce drug resistance and severe side effects, combination therapy using low-dose anticancer agents that reduce both drug resistance and side effects may be more effective for patients with HCC.

2-methoxyestradiol is an endogenous estrogen metabolite, which is produced in liver during the reproductive years of the female. It has been reported that 2-methoxyestradiol has potent anticancer and antiangiogenesis effects (3). The principal pharmacologic action of 2-methoxyestradiol is the disturbance of the function of microtubules, which then leads to cell cycle arrest at G2/M-phase (4,5). Additionally, 2-methoxyestradiol was found to inhibit superoxide dismutases (SOD) in leukemia cells (6). Inhibition of SOD causes accumulation of cellular superoxide radical and leads to free-radical-mediated damage to cells. We
have hypothesized that the low HCC incidence of females may have resulted from the higher level of 2-methoxyestradiol in the liver that inhibited or delayed the growth of HCC (7). Using an in vitro model, we have shown that 2-methoxyestradiol inhibited cell growth and induced apoptosis in human hepatoma cells. In addition, we also observed that 2-methoxyestradiol induced not only cell cycle arrest at G2/M phase but also accumulation of cellular ROS in human hepatoma cells (8). It is very likely that 2-methoxyestradiol-induced increase of free radicals contributed to cytotoxicity of hepatoma cells. However, whether the combination of 2-methoxyestradiol with other dietary flavonoids is more effective in growth inhibition of tumor cells is not clear.

Quercetin, a bioflavonoid, is widely distributed in plants and fruits, and chief dietary sources of quercetin is from apples, onions, and tea. Quercetin has been found to have many biological activities including antioxidant and prooxidant effects and cytotoxicity. About antioxidant activity, quercetin has been reported to induce elevation of glutathione (GSH)-glutamylcysteine synthetase in human hepatoma HepG2 cells (9). In contrast, Ramos and Aller (10) showed that quercetin decreased the intracellular GSH level and increased mitochondria membrane potential. Quercetin enhanced the apoptosis effect of the chemotherapeutic agent, arsenic trioxide, in leukemia cells. In addition, it was found that quercetin induced apoptosis in leukemia and pancreatic cancer cells (11,12). The quercetin-induced apoptosis in G1-S phase of tumor cells was dose dependent (13). In our previous studies, we have found quercetin inhibited the growth of hepatoma cells in dose- and time-dependent manners. Quercetin is not only effective as a single agent but also enhanced the cytotoxic effect of the chemotherapeutic agent, paclitaxel, in HA22T/VGH cells (14). These studies together have indicated that quercetin has a potential compound for cancer therapy.

2-methoxyestradiol level is high in female liver during their reproductive period. 2-methoxyestradiol or quercetin treatment alone disturbed cell cycle and increased cellular ROS content in human hepatoma cells. However, whether combination of 2-methoxyestradiol and quercetin has synergistic or additive cytotoxic effect in human hepatoma cells is unknown. The objective of this study was to explore the potential of using quercetin for increasing cytotoxic response of 2-methoxyestradiol in human hepatoma cells and to further understand the detailed mechanism of action. This study demonstrated that a combination of quercetin with 2-methoxyestradiol enhanced the cytotoxic activity in HA22T/VGH and HepG2 cells as compared to that of quercetin or 2-methoxyestradiol alone. These results suggest that combination of quercetin and 2-methoxyestradiol may have potential clinical applications.

**MATERIALS AND METHODS**

**Cell Culture**

Two human hepatoma cell lines HA22T/VGH and HepG2 were cultured in DMEM medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% gentamycin (GIBCO). Cells were kept in a humidified CO₂ incubator at 37°C with 95% air and 5% CO₂.

**Drug Treatment**

Quercetin (Sigma, St. Louis, MO) and 2-methoxyestradiol (Calbiochem Inc., San Diego, CA) were dissolved in DMSO, and the final concentrations were between 40 and 80 and 2.5 and 10 μM, respectively. The final concentration of DMSO was 0.1%. Cells were seeded and cultured for 24 h and then replenished with medium containing drugs for another 24 to 72 h and harvested for further analysis.

**Assessment of Cell Growth Inhibition**

Cells were stained with 0.4% trypan blue solution (Sigma), and viable cells were counted with a hemocytometer under a light microscope.

**Annexin V Binding Assay**

Cells were stained with annexin-V-FITC for binding with phosphatidylserine on the cell surface as an indicator of apoptosis according to the manufacturer’s instruction. Briefly, 5 × 10⁵ cells were collected, washed with binding buffer (Serotec, Oxford, UK), and then resuspended in 195 μl of the binding buffer containing 5 μl of annexin-V-FITC. After 10 min of incubation at room temperature in the dark, the cells were washed with binding buffer, resuspended in 200 μl of binding buffer containing 0.2 μg PI, and then analyzed by a flow cytometer. Data acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton Dicknison) with CellQuest software. Cells positively stained by annexin-V-FITC were considered as early apoptosis. The presence of viable (annexin V-negative and PI-negative), early apoptotic (annexin V-positive, PI-negative), and late apoptotic (annexin V-positive and PI-positive) cells were recorded. The extent of apoptosis was quantified as percentage of annexin V-positive and PI-negative cells.

**Cell Cycle Analysis**

Cycle TEST PLUS DNA Reagent Kit (Beckton Dickinson, San Jose, CA) was used for staining of DNA. After washing the cells twice with buffer solution, the cell concentration was adjusted to 1 × 10⁶/ml, and 0.5 ml of cell suspension was centrifuged at 400 g for 5 min at room temperature (20°C–25°C). The cell pellet was added with 250 μl of solution A (trypsin buffer) and gently mixed. After incubation at room temperature for 10 min, 200 μl of solution B (trypsin inhibitor and ribonuclease buffer) was added to each tube, gently mixed, and then incubated at room temperature for 10 min. This was followed with the addition of 200 μl of solution C (propidium iodide (PI) stain solution) and incubated for 10 min in the dark at 4°C. Cells were filtered through a 50 μm nylon mesh and used for flow cytometric analysis.
Flow Cytometry

Cells (20,000) were analyzed on a FASCAlibur flow cytometer (Becton Dickinson) using an argon-ion laser (15 mW) with the incident beam at 488 nm. The red fluorescence (PI) was collected through a 585 nm filter (FL-2) for cell cycle analysis. The green fluorescence (FITC) was collected through a 530 nm filter (FL-1), and red fluorescence (PI) was collected through a 650 nm filter (FL-3) for annexin V binding assay. The data were analyzed using Cellquest and ModFit software on a Macintosh computer.

Western Blot Analysis

Total proteins from cell pellets were extracted with lysis buffer [20 mM Tris buffer (pH 7.5), 1 mM EDTA, 100 µM phenylmethylsulfonyl fluoride, aprotinin (2 µg/ml), and leupeptin (2 µg/ml)]. Protein quantity was measured by Bradford assay, and an equal quantity of total protein (about 15 µg) was applied on 10% or 12.5% SDS-PAGE. After protein transfer to nitrocellulose membrane, specific protein expression level was measured using antibodies for β-actin (Sigma), MnSOD (Upstate, Albany, NY), and catalase (Upstate), respectively.

Superoxide Measurement

In order to determine the drug-induced changes in superoxide contents in the hepatoma cells, the method of Carter et al. (15) was used. Cells were washed twice with sterile Hank’s balanced salt solution (HBSS), then resuspended in sterile-filtered HBSS solution with Ca2+ and Mg2+ (GIBCO, Grand Island, NY) containing 0.22% glucose, 2 mM glutamine, and 1% bovine serum albumin. Cells were added with 10 µM hydroethidine (Molecular Probe, Eugene, OR) and incubated in 37°C for 30 min. At the end of incubation, cell suspensions were kept in an ice bath (4°C). Samples were analyzed by flow cytometer. Data are expressed as mean fluorescence of ethidium.

Mitochondrial Membrane Potential Measurement

Because mitochondria are a major source of superoxide production, we reasoned that inhibition of cell growth by quercetin and 2-methoxyestradiol might cause mitochondrial damage owing to free-radical attack on the membrane phospholipids. Alterations in the mitochondrial membrane potential were analyzed by flow cytometry using the mitochondrial membrane potential sensitive dye JC-1 (Molecular Probes, Eugene, OR), which forms monomers (FL-1) at a low membrane potential or J-aggregates (FL-2) at a higher membrane potential (16). Harvested cells were washed once with PBS, resuspended in medium containing 10 µg/mL JC-1, incubated at 37°C for 10 min, and then held at 4°C until assayed by flow cytometry. Forward scatter (FSC) versus side scatter (SSC) was used to gate the major population of cells. JC-1 monomers emit at 530 nm (FL1-H), and J-aggregates emit at 585 nm (FL2-H). Mitochondrial depolarization is indicated by the changes of fluorescence intensity.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of SOD mRNA Expression

Cells were plated at a density of 1 × 10^6 cells per 100 mm dish and were harvested at 2 days after treatment. Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation. First strand complementary DNA was synthesized from 5 µg total RNA using a reverse transcriptase (Fermentas Life Sciences, Hanover, MD) with random primer dN6 and Oligo(dT)12. Real-time quantitative PCR (qPCR) was performed using the ABI PRISM 7700 (PE Applied Biosystems, Foster City, CA), and results were analyzed with the accompanying software: SDS version 1.9.1. The DNA-intercalating SYBR green reagent (PE Applied Biosystems) was used for detection of the PCR product. PCR amplification was subsequently performed with the following primer sets: for Cu/ZnSOD, sense was 5′-CAGTCGACGTCCTCAGT-3′ and antisense was 5′-CCTGCTTTGTACTCTCTT-3′; for MnSOD, sense was 5′-CACCACGCAGCTGGCTGCC-3′ and antisense was 5′-TCCACACCCTTGGGCTGAGG-3′; for β-actin, sense was 5′-TGGCATGCGACAGGAT-3′ and antisense was 5′-GCTCAGGAGGCAATGATCT-3′. The PCR cycle used was as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The level of gene expression was obtained by the ΔΔ Ct method in which all samples were first normalized to the level of β actin in each sample. Relative expression folds were then normalized with vehicle control.

SOD Activity Assay

Whole cell lysate was prepared by resuspending the cells in M-PER protein extraction reagent (PIERCE, Rockford, IL). The superoxide dismutase activity was determined by superoxide dismutase assay kit (Cayman Chemical, Ann Arbor, MI). An aliquot of sample (10 µl) or SOD standard were pipetted into microplate wells containing 200 µl of freshly prepared reaction buffer. The reaction was initiated by the addition of 20 µl of diluted xanthine oxidase, and the optical absorbance of reduction reaction was measured at 450 nm. Cellular SOD activities are expressed in fold of their respective control.

Statistical Analysis

Data were expressed as mean ± SE (or SD). Between-group comparisons were analyzed by ANOVA. P < 0.05 was considered a significant difference.

RESULTS

Growth Inhibition of Hepatoma Cells After Treatment With Quercetin and/or 2-Methoxyestradiol

Cells were exposed to quercetin (40 µM), 2-methoxyestradiol (5 µM), or quercetin in combination with 2-methoxyestradiol for 24, 48, and 72 h. As shown in Fig. 1,
FIG. 1. The growth inhibition effects of combination treatment with 5 \( \mu \)M 2-methoxyestradiol (2-ME) and 40 \( \mu \)M quercetin (Qu) were increased significantly \( (P < 0.05) \) as compared with treatment with 2-ME alone in human hepatoma HA22T/VGH for 72 h and HepG2 cells for 48 h.

The growth inhibition effects of combination treatment with 2-methoxyestradiol and quercetin were significantly \( (P < 0.05) \) increased as compared to that of 2-methoxyestradiol alone for 48 h in human hepatoma HepG2 cells. The cells of the combination treatment groups of HA22T/VGH was significantly \( (P < 0.05) \) inhibited compared with the 2-methoxyestradiol group at 72 h.

Combination of Quercetin With 2-Methoxyestradiol Treatment Induced Apoptotic Effect in Hepatoma Cells

We examined whether the combination of quercetin with 2-methoxyestradiol treatment could enhance the apoptotic effect in HA22T/VGH and HepG2 cells using annexin V binding assay. As shown in Fig. 2, the percentage of the annexin V (+) and PI (-) cells of both hepatoma cell lines at 72 h, but not 24 or 48 h (data not shown), were significantly \( (P < 0.05) \) increased in the combination groups (16.0 ± 0.9% for HA22T/VGH; 14.6 ± 2.0% for HepG2) than either control groups (1.4 ± 0.4% for HA22T/VGH; 1.1 ± 0.1% for HepG2) or quercetin (4.8 ± 1.1% for HA22T/VGH; 2.3 ± 1.8% for HepG2) or 2-methoxyestradiol alone groups (2.6 ± 0.6% for HA22T/VGH; 1.4 ± 0.9% for HepG2).

The cell cycles were also examined at 72 h. Fig. 3 shows that treatment of hepatoma cells with 40 \( \mu \)M quercetin in combination with 5 \( \mu \)M 2-methoxyestradiol for 72 h resulted in an increase in the apoptotic subG0-G1 phase cells (22.3%) as compared to quercetin (2.3%) or 2-methoxyestradiol alone (9.7%) treatment in HA22T/VGH cells. For HepG2 cells, however the percentage of cells in subG0-G1 of the combination group (11.2%) was only slightly higher than the 2-methoxyestradiol group (3.4%); but the percentage of cells in G2-M of the combination group was increased to 43.2%, which was much more than the quercetin (25.0%) or 2-methoxyestradiol groups (8.2%).

Effect of Quercetin and 2-Methoxyestradiol on the Levels of Superoxide

To determine whether ROS is involved in the quercetin and 2-methoxyestradiol treatment induced apoptosis, we examined the treated cells using hydroethidine as probes for flow cytometric analysis of superoxide. In HA22T/VGH cells, we found that the relative mean fluorescence intensity of ethidium of the combination group (154.6% ± 4.5%) was higher than the quercetin (133.1% ± 1.1%) or the 2-methoxyestradiol group (86.8% ± 0.4%) at 48 h (data not shown), and similar results were observed up to 72 h (196.0% ± 5.0% for the combination group, 145.4% ± 19.6% for the quercetin group, and 113.8% ± 17.5% for the 2-methoxyestradiol group). Similarly, in HepG2 cells, the percentage of relative mean fluorescence of ethidium of the combination group (310.1% ± 8.0%) was increased more than the quercetin (230.8% ± 13.5%) or the 2-methoxyestradiol group (113.8% ± 17.5%) at 72 h in HepG2 cells (Fig. 4).

Expression Level of SOD and Catalase and Activity of SOD in Quercetin and 2-Methoxyestradiol Treated HA22T/VGH Cells

SODs are essential enzymes that eliminate superoxide and protect cells from free radical induced damage. To assess whether the accumulation of superoxide in treated cells was related to their SOD protein level, the treated cells were analyzed by immunoblotting with anti-MnSOD antibody. The protein expression level of MnSOD was increased in quercetin in combination with 2-methoxyestradiol as compared to quercetin or 2-methoxyestradiol alone-treated HA22T/VGH cells but not in HepG2 cells (Fig. 5). Catalase was also an antioxidant enzyme that breaks down H2O2 to H2O and an inactive compound to reduce generation of ROS. However, protein expression level
FIG. 2. The levels of annexin V binding of combination treatment with 5 \( \mu \)M 2-methoxyestradiol (2-ME) and 40 \( \mu \)M quercetin (Qu) for 72 h were significantly \((P < 0.05)\) increased as compared with treatment of either 2-ME or Qu alone in human hepatoma HA22T/VGH cells. Dimethyl sulfoxide was used as vehicle control. Data points represent mean \( \pm \) SE from triplicate of percentage of propidium iodide (PI) negative and annexin V fluorescein isothiocyanate (FITC) positive cells.

We also examined the effects of 2-methoxyestradiol and/or quercetin treatment on regulating Cu/ZnSOD and MnSOD mRNA expression by real time RT-PCR analysis. Figure 6 shows that Cu/ZnSOD mRNA expression was not altered by either quercetin, 2-methoxyestradiol alone, or combination treatments. In contrast, MnSOD mRNA level was significantly increased up to 1.5-fold after treatment with quercetin or 2-methoxyestradiol alone. 2-Methoxyestradiol and quercetin combined treatment enhanced the expression of MnSOD mRNA to 2.5-fold. These results indicated the combination treatment with quercetin and 2-methoxyestradiol upregulated MnSOD transcription in HA22T/VGH cells.

To further investigate the effect of treated groups on the SOD activity of cells, we measured SOD activity using the superoxide dismutase assay kit. Treatment of quercetin in cells significantly increased 1.81- and 1.88-fold of SOD activity as compared with control group at 48 and 72 h, respectively (Fig. 6), but treatment of 2-methoxyestradiol only had no significant effect in cells. In addition, combination treatment of quercetin and 2-methoxyestradiol in cells also significantly increased 2.78- and 2.24-fold of SOD activity at 48 and 72 h, respectively. The combination treatment group induced a higher level of SOD activity than treatment of the quercetin only group. These results showed that the increased SOD activity resulted mainly from the quercetin treatment in hepatoma cells.
FIG. 3. The effect of quercetin (Qu) and 2-methoxyestradiol (2-ME) on cell cycle progression at 72 h in HA22T/VGH and HepG2 cells. Cells were treated with 40 µM Qu, 5 µM 2-ME alone, or Qu in combination with 2-ME for 72 h, and then their cell cycle was analyzed. The representative cell cycle progressions in treated cells are from one of three independent experiments. M1, means % of cells population in sub-G0/G1.

Quercetin and 2-Methoxyestradiol Induced Reduction of Mitochondrial Membrane Potential in Hepatoma Cells

Because mitochondria are a major source of superoxide production and played an important role in cell apoptosis, we reasoned that the increase of intracellular superoxide level and cell apoptosis after treatment of quercetin and 2-methoxyestradiol might be associated with mitochondrial damage owing to free-radical attack on the membrane phospholipids. Moreover, the activation of apoptosis cascades may be linked to disruption of the mitochondrial membrane potential (17). We determined mitochondrial membrane potential alteration at 48 h after quercetin and 2-methoxyestradiol treatment in hepatoma cells. As shown in Fig. 7, the percentage of cells with lower mitochondrial membrane potential (monomeric JC-1) of the combination groups was increased as early as 48 h. For HA22T/VGH cells, the percentage of cells in monomeric JC-1 of the combination group was 19.4%, whereas the quercetin and 2-methoxyestradiol groups were 13.5% and 9.6%, respectively. For HepG2 cells, the combination group was 24.3%, whereas the quercetin and 2-methoxyestradiol groups were 14.9% and 9.8%, respectively. These results indicate that the combination treatment with quercetin and 2-methoxyestradiol led to loss of mitochondrial membrane potential.

DISCUSSION

This study demonstrated that 2-methoxyestradiol and quercetin induced apoptosis through generation of ROS in human hepatoma cells. In this study, our data showed that ROS level was increased in cells treated with quercetin and 2-methoxyestradiol. ROS production might be an important factor involved in the anticancer effect of quercetin and 2-methoxyestradiol. Recent studies have demonstrated that anticancer effect of 2-methoxyestradiol might be correlated with ROS generation (18–20). Our previous study indicated that quercetin induced apoptosis through reactive oxygen species (ROS) production and enhanced the apoptotic effect of the anticancer drug paclitaxel in human hepatoma cells (14). Combination treatment induced apoptosis significantly in human hepatoma cell lines as compared with quercetin or 2-methoxyestradiol alone.

SODs are important antioxidant enzymes for ROS metabolic processes. It was reported that 2-methoxyestradiol stimulated MnSOD activity and decreased superoxide anion level in swine granulose cells (21). Moreover, 2-methoxyestradiol increased ROS formation and inhibited growth of sarcoma cells (22). Other studies have indicated that 2-methoxyestradiol is an SOD inhibitor and decreased cancer cell survival (23, 24). In contrast,
FIG. 4. The effect of quercetin (Qu) and 2-methoxyestradiol (2-ME) or combination treatment with 5 µM 2-ME and 40 µM Qu on the production of superoxide radicals in HA22T/VGH cells for 72 h. The production of superoxide in vehicle controls and treated cells were monitored by ethidium fluorescence and analyzed by flow cytometry as described in Materials and Methods. Values are mean ± SE of the results from 3 separate experiments.

Kachadourian et al. (25) showed that 2-methoxyestradiol could not inhibit SOD activity, but 2-methoxyestradiol does increase superoxide generation in human leukemia HL-60 cells. In this study, there was no significant difference on SOD activity in cells treated with 2-methoxyestradiol alone (Fig. 6), and these data supported the Kachadourian’s (25) study. The combination treatment of 2-methoxyestradiol and quercetin in hepatoma cells resulted in significantly increased ROS level and SOD activity. Previous studies have shown that quercetin induced melanoma cells apoptosis through generation of ROS and inhibition of glutathione (26,27). Therefore, the anticancer effect of quercetin might be through ROS generation not inhibition of SOD activity and might be through the glutathione pathway.

Regarding the involvement of ROS in the antitumor effect of quercetin and 2-methoxyestradiol, previous studies have shown that ROS was associated with cell proliferation and tumor malignancy (28,29). ROS promoted cell transformation via activation of oncogene ras or epidermal growth factor receptor that enhanced cell proliferation. It also was a mediator in tyrosine kinase signal transduction pathway (30). On the other hand, ROS could cause tumor cells apoptosis by changing mitochondrial permeability (31). It was reported that quercetin enhanced melanoma cells apoptosis through generation of ROS by selectively decrease of glutathione (27); quercetin also could generate reactive quinine species and free radicals to increase cytotoxicity (26). Our data indicated that quercetin induced
FIG. 5. The expression of mitochondrial superoxide dismutase (MnSOD) in HA22T/VGH and HepG2 cells. Cells were treated with 40 µM quercetin (Qu), 5 µM 2-methoxyestradiol (2-ME) alone, or Qu in combination with 2-ME for 48 to 72 h, and then the MnSOD was analyzed by Western blotting as described in Materials and Methods. The representative MnSOD expression in treated cells is from one of two independent experiments.

FIG. 6. Real-time quantitative polymerase chain reaction (RT-PCR) analysis and superoxide dismutase (SOD) activity assay examination of 2-methoxyestradiol (2-ME) and quercetin (Qu) treatment effects on SOD alteration in HA22T/VGH cells. Real time quantitative RT-PCR analysis of the level of relative expression of Cu/Zn SOD and MnSOD mRNA in 2-ME- and Qu-treated HA22T/VGH cells. Data are presented as mean ± SD from at least three independent experiments. The SOD activity was determined by SOD assay kit in HA22T/VGH cells after treatment with Qu and/or 5 µM 2-ME for 48 to 72 h. Data are presented as mean ± SD from two independent experiments.
cells apoptosis via increase of ROS level, and quercetin combined with 2-methoxyestradiol additively enhanced anticancer activity. Therefore, quercetin-enhanced 2-methoxyestradiol anticancer effects mediated ROS production. The detail mechanism about the increase of ROS production by quercetin combined with 2-methoxyestradiol in hepatoma cells still needs further investigated.

Mitochondria generated most ROS through respiratory electron transport chain in mammalian cells. When mitochondria were damaged, ROS produced at complex I and III of the electron transport chain, which were the major intracellular sources of ROS generation in cells (32). It has been demonstrated that quercetin enhanced cisplatin-induced apoptosis in non-small-cell lung cancer cells and human head and neck cancer cells by triggering mitochondrial associated proapoptotic factors (33,34). Moreover, quercetin also induced apoptosis in human liposarcoma cells through loss of mitochondrial membrane potential (35). 2-methoxyestradiol has been shown to induce cancer cells apoptosis by stimulation of ROS production and disruption of mitochondrial membrane potential, resulting in release of cytochrome c (22,36,37). The combination of quercetin and 2-methoxyestradiol treatment resulted in a mitochondrial membrane potential loss in both HA22T/VGH and HepG2 cells (Fig. 7). Therefore, quercetin and 2-methoxyestradiol increased ROS level might be associated with disruption of mitochondrial membrane potential, and quercetin enhanced the mitochondrial membrane potential loss of 2-methoxyestradiol-treated cells. A decline of the mitochondrial membrane potential may be an early event in the process of cell death. These results suggest that initiation of growth inhibition and apoptosis of both hepatoma cells by quercetin and 2-methoxyestradiol is associated with changes in mitochondrial membrane potential.

The products of SOD action is hydrogen peroxide which can generate hydroxyl radical. A recent study indicated that quercetin increased SOD activity (38), and our results also demonstrated quercetin enhanced SOD activity in HA22T/VGH cells (Fig. 6). Catalase was also an antioxidant enzyme that breaks down H$_2$O$_2$ to H$_2$O and inactive compound to reduce generation of ROS. It was reported that increase of catalase inhibited cell proliferation and reverted tumor malignancy in human breast cancer (39). In this study, we also determined catalase protein level by Western blot on treated cells. However, protein expression level of catalase had no significant difference in each group (data not shown). We found that quercetin
combined with 2-methoxyestradiol significantly increased MnSOD mRNA expression and SOD activity (Fig. 6). It is interesting that we observed an increase in MnSOD level concomitantly with an increase in O2 production in HA22T/VGH cells. Although SOD directly controlled cellular content of ROS, SOD gene expressions were also controlled by the increase of ROS formation (40). Quercetin treatment generated ROS, which might be an important effect involved in the anticancer effect of quercetin combined with 2-methoxyestradiol. Our results agree well to the finding that ROS generation agent manumycin enhanced the antioxidant effect of 2-methoxyestradiol (20). Therefore, the quercetin-enhanced cell apoptosis appears to be mediated through increase of ROS stress, but the contribution of hydroxy radical in this process is worthy of further investigation.

2-methoxyestradiol is a metabolite of estradiol in women, and quercetin is a nature compound that widely exists in plants and fruits. Interestingly, our data demonstrated that the generation of ROS correlated well with the combination treatment to inhibit tumor cell growth and induce cell apoptosis. 2-methoxyestradiol-induced apoptosis was well correlated with an increase in ROS (20). Combined with quercetin could enhance 2-methoxyestradiol-induced anticancer effect. Although the detail mechanism involved in anticancer activity needs further investigation, this study provided evidence that quercetin increased the apoptosis action of 2-methoxyestradiol in hepatoma cells and raised the possibility of flavonoids to improve the cytotoxicity of anticancer drugs.

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