Resveratrol Reduces the Hypoxia-Induced Resistance to Doxorubicin in Breast Cancer Cells

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Summary Resveratrol (3,4’,5-trihydroxy-trans-stilbene) is known to enhance the cytotoxicity of the anticancer drug doxorubicin. On the other hand, breast cancer MCF-7 cells acquire resistance to doxorubicin under hypoxic conditions. In this study, we investigated the effect of resveratrol on hypoxia-induced resistance to doxorubicin in MCF-7 cells. Resveratrol and its derivative 3,5-dihydroxy-4’-methoxy-trans-stilbene, but not 3,5-dimethoxy-4’-hydroxy-trans-stilbene, cancelled hypoxia-induced resistance to doxorubicin at a concentration of 10 μM. Carbonyl reductase 1 (CBR1) catalyzes the conversion of doxorubicin to its metabolite doxorubicinol, which is much less effective than doxorubicin. Hypoxia increased the expression of CBR1 at both mRNA and protein levels, and knockdown of CBR1 inhibited hypoxia-induced resistance to doxorubicin in MCF-7 cells. Knockdown of hypoxia-inducible factor (HIF)-1α repressed the hypoxia-induced expression of CBR1. Resveratrol repressed the expression of HIF-1α protein, but not HIF-1α mRNA, and decreased hypoxia-activated HIF-1 activity. Resveratrol repressed the hypoxia-induced expression of CBR1 at both mRNA and protein levels. Likewise, 3,5-dihydroxy-4’-methoxy-trans-stilbene decreased the hypoxia-induced expression of CBR1 protein, but not 3,5-dimethoxy-4’-hydroxy-trans-stilbene. Furthermore, resveratrol decreased the expression of HIF-1α protein even in the presence of the proteasome inhibitor MG132 in hypoxia. Theses results indicate that in MCF-7 cells, HIF-1α-increased CBR1 expression plays an important role in hypoxia-induced resistance to doxorubicin and that resveratrol and 3,5-dihydroxy-4’-methoxy-trans-stilbene decrease CBR1 expression by decreasing HIF-1α protein expression, perhaps through a proteasome-independent pathway, and consequently repress hypoxia-induced resistance to doxorubicin.

Key Words resveratrol, doxorubicin, carbonyl reductase 1, hypoxia-inducible factor-1α, breast cancer cells

Breast cancer is the most frequently diagnosed carcinoma and the second leading cause of cancer death in women (1). Solid tumors grow in a hypoxic environment resulting from insufficient oxygen supply due to dysfunctional tumor vessels. Hypoxia not only promotes the growth of a tumor by enhancing glycolytic flux and increasing angiogenesis, but also allows tumors to acquire resistance to anticancer drugs (2, 3). Recently, the uses of functional foods with antioxidants and anti-cancer activities have emerged as a promising strategy for the treatment of various diseases including cancer (4–6). However, little is known about the possible interactions between functional foods and chemotherapeutic agents.

Resveratrol (3,4’,5-trihydroxy-trans-stilbene) is a phytopolyphenol and is present in a variety of edible plant products including peanuts, berries, and grape skin (7). Resveratrol exerts preventive effects against pathological processes such as inflammation (8), atherosclerosis (9), cardiovascular disease (10), type 2 diabetes (11), and tumorigenesis including breast cancer (12). Furthermore, resveratrol enhances the cytotoxic effect of the anticancer drug doxorubicin on the growth of breast cancer MCF-7 cells (13, 14). However, its molecular mechanisms remain unclear. Recently, we found that resveratrol inhibits enzymatic activity of carbonyl reductase 1 (CBR1), a doxorubicin-metabolizing enzyme, by directly binding to CBR1 (14).

CBR1 is an NADPH-dependent enzyme belonging to the short chain dehydrogenase family and catalyzes a large number of biologically and pharmacologically active substrates, including endogenous substrates such as prostaglandin E2 and S-nitrosoglutathione and xenobiotic substrates such as anthracycline anti-
cancer drugs (doxorubicin and daunorubicin) (15–17). Since doxorubicin causes DNA damage by intercalating itself into the DNA base pairs of the double helix, it is used for the treatment of broad range of solid tumors, including breast cancer and hepatocellular carcinoma cells (18, 19). However, CBR1 metabolizes the reduction of doxorubicin to doxorubicinol, which is less potent at killing tumor cells and which induces cardiotoxicity (20). The CBR1 activities in lung and breast cancer tissues are higher than those in the corresponding normal tissues (21). Furthermore, hypoxia enhances resistance to doxorubicin in tumor cells and increases the expression of CBR1 through hypoxia-inducible factor (HIF)-1α in hepatocellular carcinoma cells (22). These results indicate that CBR1 plays a role in the acquisition of resistance to doxorubicin and that the clinical application of doxorubicin is limited because it has a risk of cardiotoxicity.

In this study, we assessed the combined effects of resveratrol and doxorubicin on the growth of MCF-7 cells in hypoxia. We demonstrate that resveratrol suppresses hypoxia-induced resistance to cytotoxicity of doxorubicin and represses the expression of CBR1 in MCF-7 cells. Furthermore, we report that resveratrol inhibits CBR1-mediated resistance to doxorubicin by decreasing the expression of HIF-1α in hypoxia.

MATERIALS AND METHODS

Reagents. Resveratrol and 3,5-dimethoxy-4′-hydroxy-trans-stilbene were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and 3,5-dihydroxy-4′-methoxy-trans-stilbene was synthesized according to the method of Paul et al. (23). Doxorubicin hydrochloride and doxorubicinol hydrochloride were purchased from Enzo Life Sciences (Farmingdale, NY) and Toronto Research Chemicals (Toronto, Canada), respectively. Hoechst 33342 was obtained from Dojindo Laboratories (Kumamoto, Japan).

Cell culture. Human breast cancer MCF-7 cells were obtained from the RIKEN BioResource center (Ibaraki, Japan). The cells were cultured in Dulbecco’s modified Eagle’s medium-high glucose (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (DMEM-HG) at 37°C in a 5% CO2/95% air atmosphere at 100% humidity.

siRNA. Human HIF-1α siRNA (siHIF-1α) and control siRNA (siControl) were designed previously (24). Double-strand siRNA for human CBR1 was chemically synthesized. Target sequences for siRNA duplexes were as follows: siCBR1, 5′-CACAGAATTACTCCCTCTA-3′ (Sigma-Aldrich, Saint Louis, MO). The duplexes (10 nM) were introduced into MCF-7 cells using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) for 48 h, and the cells were incubated in fresh DMEM-HG medium in normoxia or hypoxia for an additional 24 h.

Cell viability assay. Cell survival was quantified using Hoechst 33342. MCF-7 cells (1.2×104 cells/well) were cultured in DMEM-HG on 24-well plates for 24 h, followed by incubation with various concentrations of resveratrol (0–10 μM) in the presence or absence of doxorubicin (50 nM) in normoxia or hypoxia for 72 h. The cells were lysed with phosphate-buffered saline, and cell lysates were incubated with Hoechst 33342 (0.4 ng/μL) at room temperature for 10 min. Fluorescence intensity of Hoechst 33342 was measured by fluorometer with 355/460 nm filter excitation and emission set.

Western blot. MCF-7 cells were lysed in lysis buffer (20 mM Hepes-NaOH, pH 7.4, containing 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 1 μg/mL aprotinin). The cell lysates were subjected to SDS-PAGE and analyzed by Western blotting using rabbit polyclonal anti-CBR1 (Aviva Systems Biology, San Diego, CA) antibody, rat monoclonal anti-HSP90 (Clone: 16F1, StressGen Biotechnologies, Victoria, Canada) antibody, and mouse monoclonal anti-HIF-1α (Clone: mcg3, Affinity Bioreagents, Golden, CO) and anti-α-tubulin (Clone: DM 1A, Sigma-Aldrich) antibodies. Immunoreactive proteins were incubated with horseradish peroxidase-conjugated secondary antibodies and reacted with Immobilon Western Chemiluminescent HRP-Substrate (Millipore, Billerica, MA), followed by detection with an LAS4000 imaging system (GE Healthcare, Upssala, Sweden).

Semi-quantitative RT-PCR. Total RNAs were extracted from MCF-7 cells with Sepasol-RNA Super (Nacalai Tesque, Inc., Kyoto, Japan), and cDNAs were synthesized by reverse transcriptase. The nucleotide sequences of primers for HIF-1α and β-actin were described previously (24). The primers for CBR1 were designed (forward primer: 5′-TGTTCCGGCATCATGTA-3′ and reverse primer: 5′-CCACTGTTCAACTCTCTTCT-3′).

Luciferase reporter assay. MCF-7 cells were grown on 48-well plates in DMEM-HG and transiently transfected with reporter vector (pEpo-HRE-Luc (24) and pRL-SV40 (Promega, Madison, WI)) using HilyMax reagent (Dojindo Laboratories) for 24 h. The medium was replaced with fresh DMEM-HG, and the cells were incubated in the presence of various concentrations (0–25 μM) of resveratrol in normoxia or hypoxia for 24 h. Luciferase reporter activities were determined using a dual-luciferase reporter assay system (Promega). Data are expressed as relative light units (RLU, firefly luciferase activity divided by Renilla luciferase activity).

Statistical analysis. Data were assessed by two-way analysis of variance with Tukey’s post hoc testing. Statistical analysis was performed using JMP statistical software version 8.0.1 (SAS Institute, Cary, NC). Experimental values are expressed as mean±SD, and statistically significant differences (p<0.05) are indicated by different letters.

RESULTS

To examine the effect of doxorubicin on cell viability under hypoxic conditions in breast cancer cells, MCF-7 cells were incubated with various concentrations of doxorubicin. Doxorubicin decreased cell viability of MCF-7 cells in a dose-dependent manner, and hypoxia attenuated the cytotoxicity of doxorubicin at concentrations above 10 nM (Fig. 1A). We assessed the inhibitory effect of resveratrol on hypoxia-induced resistance
Resveratrol at a concentration of 1 μM had no influence on cell viability in the presence or absence of doxorubicin in normoxia, but attenuated hypoxia-induced resistance to doxorubicin (Fig. 1B). On the other hand, resveratrol at a concentration of 10 μM completely canceled hypoxia-induced resistance to cytotoxicity of doxorubicin. Next, we determined the effects of resveratrol and its derivatives on the hypoxia-induced resistance to doxorubicin. Resveratrol and 3,5-dihydroxy-4′-methoxy-trans-stilbene (4′-OMe) at a concentration of 10 μM suppressed the viability of MCF-7 cells in hypoxia and inhibited the hypoxia-induced resistance to doxorubicin (Fig. 1D). In contrast, 3,5-dimethoxy-4′-hydroxy-trans-stilbene (3,5-OMe) had no influence on the cell viability or the resistance to doxorubicin in hypoxia.

To determine whether CBR1 is involved in hypoxia-induced resistance to doxorubicin, MCF-7 cells were transiently transfected with siRNA for CBR1, followed by incubation with doxorubicin in normoxia or hypoxia. Hypoxia increased the expression of CBR1 at both the mRNA and protein levels (Fig. 2A). In both normoxia and hypoxia, siRNA for CBR1 suppressed the expression of CBR1, but not the expressions of β-actin or α-tubulin. Knockdown of CBR1 restored hypoxia-induced resistance to cytotoxicity of doxorubicin (Fig. 2B). On the other hand, siCBR1 had no influence on cell viability in the absence of doxorubicin.

To investigate whether HIF-1α is involved in hypoxia-induced expression of CBR1, HIF-1α was knocked down using siRNA for HIF-1α in MCF-7 cells, and then the expression level of CBR1 was assessed. Knockdown of HIF-1α canceled the hypoxic induction of the expressions of HIF-1α and CBR1 (Fig. 3A). Next, we determined the effects of resveratrol on the expression of CBR1 and HIF-1α in hypoxia. Resveratrol at concentrations above 10 μM decreased the expression level of HIF-1α protein, but not of HIF-1α mRNA (Fig. 3B). In contrast, resveratrol at concentrations above 10 μM reduced the expression of CBR1 at both mRNA and protein levels. Resveratrol reduced hypoxia-activated HIF-1 activity in a dose-dependent manner (Fig. 3C). Furthermore, hypoxia-induced expression levels of HIF-1α and CBR1 protein were reduced by resveratrol and 4′-OMe, but not by 3,5-OMe (Fig. 3D).

We assessed whether proteasome is involved in res-
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veratrol-decreased expression of HIF-1α in hypoxia. A proteasome inhibitor, MG132, increased the expression level of HIF-1α protein (first and third lanes in Fig. 4) and the expression level of resveratrol-decreased HIF-1α protein (second and forth lanes in Fig. 4). However, resveratrol decreased the expression level of HIF-1α protein even in the presence of MG132 (third and forth lanes in Fig. 4). In contrast, although the expression of HSP90 is induced by MG132 (25), resveratrol had no influence on MG132-induced HSP90 expression level.

**DISCUSSION**

Recently, combination treatment with a chemotherapeutic agent and functional foods has been increasing among cancer patients (5). However, combined usage of functional foods with a chemotherapeutic agent might cause side effects or might attenuate the effect of the therapeutic agent. CBR1 could be a promising target molecule for resistance to cytotoxicity of doxorubicin, because CBR1 converts doxorubicin to doxorubicinol, which is less effective against cancer and which induces cardiotoxicity. We previously demonstrated that resveratrol directly interacts with CBR1 and inhibits its enzymatic activity (14). Therefore, resveratrol may have the potential to be used in combination with doxorubicin in the treatment of cancer. In this study, we demonstrated that resveratrol suppresses hypoxia-induced resistance to cytotoxicity of doxorubicin in MCF-7 cells. Resveratrol inhibited hypoxia-induced resistance to
doxorubicin in a concentration-dependent manner. Tumors increase or acquire resistance to doxorubicin in several ways, including (i) increased expression of drug transporters (26), (ii) enhancement of doxorubicin metabolism (27), and (iii) alteration of intracellular localization of doxorubicin (28). Doxorubicin is deactivated by several carbonyl reducing enzymes, including carbonyl reductases (e.g., CBR1 and CBR3) (29, 30) and aldo-keto reductases (e.g., AKR1A1, AKR1B10, AKR1C3, and AKR1C4) (31–33). CBR1 activities are increased in human lung and breast cancer tissues (21). AKR1C3 is predominantly expressed in human prostate and mammary gland, compared to AKR1C4, and AKR1C3 and AKR1C4, which are expressed at similar levels in the liver (33). Thus, the activities and expressions of these carbonyl reducing enzymes are different between normal cells and cancer cells and among tissues. In the present study, knockdown of CBR1 canceled hypoxia-induced resistance to doxorubicin in MCF-7 cells, and resveratrol repressed the expression of CBR1. These results indicate that CBR1 is a key factor in hypoxia-induced resistance to cytotoxicity of doxorubicin in MCF-7 cells and that resveratrol suppresses hypoxia-induced resistance to doxorubicin by decreasing the expression of CBR1.

Resveratrol decreased the expression of HIF-1α and CBR1 at concentrations above 10 μM. HIF-1α increases the expression of CBR1 at the transcriptional level in hypoxic hepatocellular carcinoma cells (22), consistent with the present results that knockdown of HIF-1α down-regulated CBR1 expression in hypoxic MCF-7 cells. Some flavonoids such as quercetin and luteolin, which are structurally similar to resveratrol, suppress hypoxia-mediated resistance to doxorubicin. Luteolin restores hypoxia-induced tumor resistance to doxorubicin in breast cancer 4T1 and MCF-7 cells (34) and decreases HIF-1α expression in hypoxic human brain astrocytes (35). Quercetin improves the therapeutic index of doxorubicin by degrading HIF-1α protein in hypoxic breast cancer 4T1 cells (36). Thus, quercetin and luteolin may decrease the CBR1 protein level by suppressing the expression of HIF-1α. Quercetin-reduced HIF-1α protein is not restored by the proteasome inhibitor in prostate cancer LNCaP cells (37). Likewise, in hypoxic MCF-7 cells, resveratrol decreased HIF-1α protein level even in the presence of MG132 (Fig. 4). Thus, resveratrol seems to decrease HIF-1α protein through a proteasome-independent pathway. In contrast, in A2780/C70 and HepG2 cells, resveratrol degrades HIF-1α protein through the ubiquitin-proteasome pathway (38, 39). Therefore, in hypoxia, the degradation pathway of HIF-1α protein by resveratrol is different depending on the cell types. Taken together, these results indicate that resveratrol suppresses CBR1-mediated resistance to doxorubicin by decreasing the HIF-1α protein, perhaps through a proteasome-independent pathway, in hypoxic MCF-7 cells.

The expression level of HIF-1α protein was decreased by resveratrol and its derivative 3,5-dihydroxy-4′-methoxy-trans-stilbene, but not by 3,5-dimethoxy-4′-hydroxy-trans-stilbene. Thus, at least the hydroxyl group of the 3 or 5 position of resveratrol plays an important role in the mechanism by which resveratrol decreases HIF-1α protein level. The 3,5-dihydroxy groups of resveratrol are required for direct interaction with CBR1 (14). Furthermore, the transcriptional activity of androgen receptor is inhibited by resveratrol and 3,5-dihydroxy-4′-methoxy-trans-stilbene, but not by 3,5-dimethoxy-4′-hydroxy-trans-stilbene (40). In contrast, the 4′-hydroxy group of resveratrol is required for enhancing the transcriptional activity of estrogen receptor α (41). Thus, the number and the position of hydroxyl groups of resveratrol seem to play an important role in interaction with resveratrol-binding proteins.

Doxorubicin-based chemotherapy has improved the survival of many patients with breast cancer (18). However, cardiotoxicity is the unexpected side effect of doxorubicinol and limits the clinical application of doxorubicin (42). Because mice with a null allele of CBR1 are protected from doxorubicin-induced cardiotoxicity (43), CBR1 is a major enzyme to produce doxorubicinol. Therefore, resveratrol is expected to down-regulate the CBR1 expression, and the combined use of doxorubicin and resveratrol is expected to decrease the effective concentration of doxorubicin (i.e., less doxorubicin will be needed if resveratrol is given also) in the treatment of breast cancer. Furthermore, resveratrol suppresses doxorubicin-induced cardiotoxicity by increasing heme oxygenase-1 expression (44). Therefore, resveratrol appears to be a promising candidate not only for enhancing the anticancer activity of doxorubicin, but also for reducing the doxorubicin-induced cardiotoxicity.

REFERENCES


