Laboratory of Clinical Pharmacy and Therapeutics[1], Faculty of Pharmacy, Osaka Ohtani University, Tondabayashi; Pharmaceutical Education Support Center[2], School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Japan

Reduced cytotoxicity in doxorubicin-exposed HepG2 cells pretreated with menthol due to upregulation of P-glycoprotein

K. Nagai[1,*], S. Fukuno[1], T. Miura[2], Y. Uchino[1], N. Sehara[1], H. Konishi[1]

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**Corresponding author: Katsuhito Nagai, Ph.D., Laboratory of Clinical Pharmacy and Therapeutics[1], Faculty of Pharmacy, Osaka Ohtani University, 3-11-1 Nishikiori-kita, Tondabayashi 584-0066, Japan*
nagaika@osaka-ohtani.ac.jp

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The aim of the present study was to examine changes in the expression and activity of P-glycoprotein (P-gp) in human hepatocellular carcinoma HepG2 cells after exposure to menthol, and their relationship to the cytotoxicity of and apoptotic responses to doxorubicin (DOX), a substrate of P-gp, in the cells. The expression of P-gp in HepG2 cells was significantly increased by menthol treatment. Intracellular accumulation of DOX in HepG2 cells was significantly lower in the menthol-treated group than in the control group, but this phenomenon was abolished in the presence of verapamil. Decreased cell viability by DOX was significantly attenuated by 24-h menthol treatment prior to DOX exposure, which coincided with the changes in mRNA expression of Bcl-xl and caspase-3. These results demonstrate that menthol causes hepatocellular carcinoma cells to acquire resistance to DOX by increasing its efflux through the upregulation of P-gp.

1. Introduction

Hepatocellular carcinoma (HCC) is a prevalent cancer of the liver, and frequently develops in patients with underlying chronic hepatic disease and cirrhosis. Approximately 80% of HCC patients are currently diagnosed at advanced stages and are not suitable candidates for surgical resection. Systemic chemotherapy using cytotoxic agents, including doxorubicin (DOX), and targeted therapy with the tyrosine kinase inhibitor sorafenib are the main approaches for these patients; however, the development of resistance to these anticancer drugs remains a major clinical obstacle (Lohitesh et al. 2018). In chemotherapy resistance, the overexpression of the drug efflux transporter P-glycoprotein (P-gp), which results in DOX tolerance, has been a major concern over the past 40 years (Fletcher et al. 2016).

Menthol, a cyclic monoterpene alcohol, is an organic compound made synthetically or obtained from the oils of several mints. Our previous study demonstrated that hepatic CYP3A2 was upregulated in menthol-treated rats, which was responsible for the alteration in pharmacokinetic behavior of the substrate drug midazolam (Nagai et al. 2015). P-gp and CYP3A were reported to have considerable overlap in inducers *in vitro* (Matheny et al. 2004), and we therefore hypothesized that menthol will render HCC resistant to anticancer drugs, such as DOX, by increasing P-gp expression. In the present study, we examined the effects of menthol on the gene expression and transport activity of P-gp in human hepatocellular carcinoma HepG2 cells. Furthermore, the change in cytotoxicity of DOX was investigated after menthol treatment.

2. Investigations and results

The mRNA expression of P-gp in HepG2 cells was significantly increased by menthol (Fig. 1A). This alteration was consistent with the intracellular accumulation of DOX being reduced by menthol in HepG2 cells (Fig. 1B). In addition, the involvement of menthol in increased P-gp function may be explained by the increased intracellular DOX level in menthol-treated HepG2 cells in the presence of verapamil, a typical P-gp inhibitor (Abdallah et al. 2015) (Fig. 1B). On evaluation of cell viability, the survival rate of HepG2 cells exposed to DOX was significantly increased when treated with menthol in advance (Fig. 2A). Overexpression of Bcl-xl, a molecule associated with intrinsic apoptosis, was reported to play a significant role in acquired resistance of HCC to anticancer drugs, including DOX (Park et al. 2007). Signal transduction through intrinsic apoptotic pathways was reported to active caspase-3, a key cell death protease (Müller et al. 1998). In the present study, the gene expression of Bcl-xl and caspase-3 in HepG2 cells exposed to DOX was significantly reduced and increased, respectively; however, this was abolished by menthol treatment in advance (Figs. 2B and 2C). Considering the lack of direct effects of menthol on the viability of HepG2 cells (Fig. 2A), the cytotoxic and apoptotic responses of HCC to DOX were suggested to have been reduced by the insufficient level of intracellular DOX through the induction of P-gp by pretreatment with menthol.

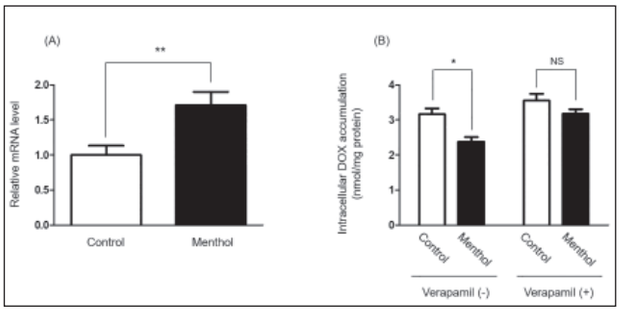

Fig. 1: Effects of menthol on the expression and activity of P-gp. (A) After total RNA of HepG2 cells was extracted, the expression level of P-gp was evaluated using real-time PCR analysis. Data were normalized to GAPDH, which was used as an internal control. (B) HepG2 cells were exposed to 10 μM DOX for 30 min in the absence or presence of 100 μM verapamil, and then intracellular DOX accumulation was measured to assess P-gp activity. The results are shown as means±SD from three independent experiments. *: $p<0.05$, **: $p<0.01$, NS: not significant.

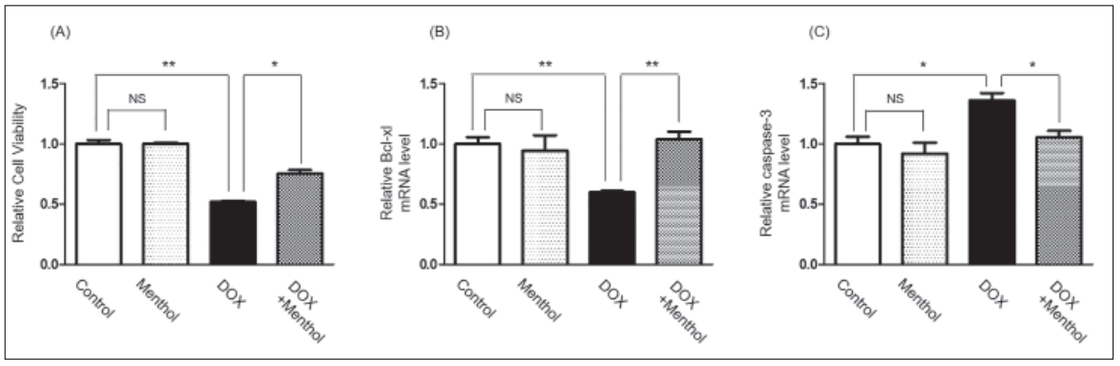

Fig. 2: Effects of menthol on cytotoxicity and apoptotic responses after exposure to DOX. (A) The cells were exposed to 10 μM DOX 24 h after treatment with 100 μM menthol, and cell viability was then evaluated by the MTT method 24 h later. (B and C) The expression levels of Bcl-xl (B) and caspase-3 (C) were evaluated using real-time PCR analysis. The results are shown as means±SD from three independent experiments. *: $p<0.05$, **: $p<0.01$, NS: not significant.

3. Discussion

Menthol is commercially available as ingredient of a number of dietary items, including functional foods and cough drops. In clinical settings, peppermint oil is also used to successfully treat irritable bowel syndrome (IBS) (Pirotta 2009). The proposed daily requirement of peppermint oil is approximately 1,100 mg for IBS. The blood concentration of menthol following the oral administration of 36-mg peppermint oil capsules to healthy male volunteers was reported to reach 10 μM (Mascher et al. 2001). Therefore, when peppermint oil capsules are therapeutically used, the blood concentration of menthol may reach 100 μM due to its efficient absorption with a bioavailability exceeding 70% (Hiki et al. 2011). This may lead to a failure of HCC treatment with DOX through P-gp upregulation in clinical practice. In addition to DOX, P-gp acts as an energy-dependent drug efflux pump of many structurally unrelated chemotherapeutic drugs such as vincristine, etoposide, and taxol (Abdallah et al. 2015). Therefore, medical staff should be required to advise HCC patients undergoing chemotherapy not to take menthol-rich supplements in order to reduce the risk of multi-drug resistance. In conclusion, the present study demonstrated that pretreatment with menthol reduces the cytotoxic activity of DOX in HepG2 cells by reducing the intracellular DOX level through the upregulation of P-gp. Our study provides useful information for the treatment of HCC using anticancer drugs.

4. Experimental

4.1. Cell treatment

For real-time PCR and transport assay for P-gp, HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were treated with 100 μM menthol for 24 h. Menthol was dissolved in dimethyl sulfoxide (DMSO).

For the evaluation of cell viability, HepG2 cells were divided into four treatment groups, which were designated as DOX, menthol, DOX plus menthol, and control. In the DOX group, 24-h treatment with DOX (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in culture medium was performed on HepG2 cells 24 h after the addition of DMSO alone. In the menthol group, menthol (Cosmo Bio Co., Ltd, Tokyo, Japan) was added again 24 h after the first addition of menthol and incubated with HepG2 cells for a total of 48 h. In the DOX plus menthol group, 24-h concurrent treatment with DOX and menthol was performed on HepG2 cells 24 h after the addition of menthol alone. Control cells were treated with vehicle alone.

For real-time PCR to assess the expression level of Bcl-xl and caspase-3, HepG2 cells were divided into four treatment groups, which were designated as DOX, menthol, DOX plus menthol, and control. In the DOX group, 3-h DOX treatment was performed on HepG2 cells 24 h after the addition of DMSO alone. In the menthol group, menthol was added again 24 h after the first addition of menthol and incubated with HepG2 cells for a total of 27 h. In the DOX plus menthol group, 3-h concurrent treatment with DOX and menthol was performed on HepG2 cells 24 h after the addition of menthol alone. Control cells were treated with vehicle alone.

4.2. Real-time PCR

Gene expression was evaluated using the real-time quantitative PCR based on our previous report (Nagai et al. 2015). The synthetic oligonucleotide primers (Hokkaido System Science Co., Ltd., Sapporo, Japan) were designed by Beacon Designer 8 (Bio-Rad Lab., Inc.). P-gp was amplified using the 5' primer 5'- AGGAG-CAAAGAAGAAGAACT-3' and 3' primer 5'-CCACCACCATATACAACTTG-3'. Bcl-xl was amplified using the 5' primer 5'-CCCAGAAAGGATACAGCTGG-3' and 3' primer 5'-GCGATCCGACTCACCAATAC-3'. Caspase-3 was amplified using the 5' primer 5'-CAAACTTTTTCAGAGGGGATCG-3' and 3' primer 5'-GCATACTGTT-TCAGCATGGCA-3'. GAPDH was amplified using the 5' primer 5'-CGTCTGCCC-TATCAACTTTCG-3' and 3' primer 5'-CGTTTCTCAGGCTCCCTCT-3'. Gene expression of the target sequence was normalized to that of GAPDH, and the results were expressed relative to control values, which were an arbitrary value of 1.

4.3. Transport assay

DOX (10 μM) was added to the cells and reacted for 30 min in Hanks' balanced salt solution in the presence or absence of 100 μM verapamil. The reaction was stopped by adding ice-cooled excess phosphate-buffered saline. Fluorescence intensity was measured using a microplate reader (SYNERGY H4; BioTek LLC, Tokyo, Japan) to evaluate the accumulated amount of DOX. The excitation and fluorescence wavelengths were 470 nm and 550 nm, respectively. Protein concentrations were measured based on the Lowry method.

4.4. Cell viability

Cell viability was evaluated using the MTT assay. After 24-h exposure to DOX, the cells were cultured for 30 min with 500 μg/mL of MTT. The cells were lysed with DMSO and the absorbance of purple formazan produced by the living cells was monitored at a wavelength of 570 nm.

4.5. Statistical analysis

Differences between the means of two groups were compared using the Student's unpaired *t*-test. The significance of differences between control and test values was determined using the two-tailed multiple *t*-test with Bonferroni correction following one-way analysis of variance (3 comparisons of 4 groups). Differences with a *p*-value of 0.05 or less were considered significant.

Conflicts of interest: None declared.

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