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# Sofalcone, a gastric mucosa protective agent, increases vascular endothelial growth factor via the Nrf2-heme-oxygenase-1 dependent pathway in gastric epithelial cells

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ABSTRACT

Sofalcone, 2′-carboxymethoxy-4,4-bis(3-methyl-2-butenyloxy)chalcone, is an anti-ulcer agent that is classified as a gastric mucosa protective agent. Recent studies indicate heat shock proteins such as HSP32, also known as heme-oxygenase-1(HO-1), play important roles in protecting gastrointestinal tissues from several stresses. We have previously reported that sofalcone increases the expression of HO-1 in adipocytes and pre-adipocytes, although the effect of sofalcone on HO-1 induction in gastrointestinal tissues is not clear. In the current study, we investigated the effects of sofalcone on the expression of HO-1 and its functional role in rat gastric epithelial (RGM-1) cells. We found that sofalcone increased HO-1 expression in RGM-1 cells in both time- and concentration-dependent manners. The HO-1 induction was associated with the nuclear translocation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in RGM-1 cells. We also observed that sofalcone increased vascular endothelial growth factor (VEGF) production in the culture medium. Treatment of RGM-1 cells with an HO-1 inhibitor (tin-protoporphyrin), or HO-1 siRNA inhibited sofalcone-induced VEGF production, suggesting that the effect of sofalcone on VEGF expression is mediated by the HO-1 pathway. These results suggest that the gastroprotective effects of sofalcone are partly exerted via Nrf2-HO-1 activation followed by VEGF production.



## 1. Introduction

Sofalcone (2′-carboxymethoxy-4,4′-bis(3-methyl-2-butenyloxy)chalcone) is an anti-ulcer agent used for the protection of gastric mucosa. This drug has been used for the treatment of gastritis or gastric ulcers in Japan and South Korea. Studies have suggested that the pharmacological efficacy of sofalcone is due to the inhibition of 15-hydroxy-prostaglandin(PG)-dehydrogenase [1], or increasing blood flow in mucosa. This drug is also known to have anti-bacterial activities against *Helicobacter pylori* (*H. pylori*) or anti-inflammatory effects on *H. pylori*-associated gastric inflammation through the inhibition of pro-inflammatory cytokine production [2,3].

Heme-oxygenase-1 (HO-1) is an inducible rate limiting enzyme, which catalyzes free heme into carbon monoxide (CO), free iron, and biliverdin. It is known that HO-1-derived CO and biliverdin have strong anti-inflammatory and anti-oxidant properties [4]. HO-1 is also known as a 32 kDa isoform among heat shock proteins (HSPs). Recent studies have shown that HSPs play important roles in protecting gastric tissues from several stimuli [5–7]. We have previously reported that sofalcone induces HO-1 protein expression in 3T3 adipocytes and pre-adipocytes [8] and that this effect is associated with adipocyte differentiation and with the amelioration of inflammatory responses between macrophages and adipocytes. However, whether sofalcone induces HO-1 protein in gastric cells or its pharmacological significance of HO-1 induction by this drug is not clear.

Vascular endothelial growth factor (VEGF) is a potent promoter of angiogenesis, which is crucial in gastric ulcer healing [9]. Deng, et al. demonstrated that VEGF gene transfer for ulcers accelerated the experimental ulcer healings [10]. It has also been reported that sofalcone increases VEGF production in gastric fibroblasts [11], although the mechanism by which VEGF production is facilitated by sofalcone has yet to be clearly shown.

Recent studies have demonstrated an association between HO-1 and VEGF production. HO-1 activation by several inducers (hemin, 15-deoxy-D12, 14-prostaglandin $J_2$ (15d-$PGJ_2$), or HO-1 gene transfer) or CO, an HO product, has been reported to lead to VEGF synthesis in vascular smooth muscle cells or endothelial cells

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[12,13]. However, the association of HO-1 induction with VEGF production in gastric cells and its implication in ulcer repair has yet to be understood.

Thus, in the current study, we investigated the effect of sofalcone on HO-1 expression in RGM-1 cells. To clarify an association of HO-1 with VEGF production in gastric cells, we also examined the effect of sofalcone on VEGF expression and the role of HO-1 in the pharmacological actions of this drug.

## 2. Materials and methods

### 2.1. Reagents

Sofalcone (2′-carboxymethoxy-4,4′-bis(3-methyl-2butenyloxy)chalcone) was kindly provided by Taisho Toyama Pharma Co., Ltd (Tokyo, Japan). Antibodies for murine HO-1 and β-actin were purchased from Stressgen (Michigan, USA) and Abcam (Tokyo, Japan), respectively. Kits for enzyme-linked immunosorbent assay (ELISA) for the determination of VEGF in culture media were purchased from R&D Systems (Tokyo, Japan). Tin-protoporphyrin (SnPP) was purchased from Frontier Scientific, Inc. (Utah, USA). These reagents were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the medium was kept at 0.2% throughout the experiments.

### 2.2. Cells and culture conditions

Rat gastric mucosal cells (RGM-1) were grown to confluence in Dulbecco's modified essential medium/Ham F12 (DMEM) (Sigma–Aldrich) containing 10% FBS at 37 °C and 5% $CO_2$.

### 2.3. Viability assay

Cell viability was determined by a WST-8 cell counting assay (Dojindo, Tokyo, Japan) according to the manufacturer's instructions.

### 2.4. Western blotting

After treatment, RGM-1 cells were harvested and lysed with a lysis buffer (Cellytic-M, Sigma) containing a protease inhibitor cocktail (Roche Applied Science, Tokyo, Japan). Protein concentration was determined by a BCA protein assay reagent (Thermo Scientific, Inc., Illinois, USA). The samples were diluted with a lysis buffer containing β-mercaptoethanol. Equal amounts of protein (10 μg) were separated on SDS–polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (Immobilong-P; Millipore, Tokyo, Japan). Proteins were detected by immunoblotting followed by ECL chemiluminescence detection (GE Biosciences, Tokyo, Japan). Chemiluminescence signals were detected by a Luminoimage Analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

### 2.5. Detection of Nrf2 nuclear translocation

Cells were cultured in 100 mm diameter dishes and nuclear proteins were extracted using an NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Protein concentration was quantified by a bicinchoninic acid (BCA) assay method (Thermo Scientific). Equivalent amounts of nuclear protein (5 μg) were separated by SDS–polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Tokyo, Japan). Proteins were detected by immunoblotting followed by ECL chemiluminescence detection (Amersham Biosciences, Buckinghamshire, England). Nrf2 (H-300, sc-13032) (Santa Cruz Biotechnology, Santa Cruz, CA) antibody was used as a first antibody. Secondary antibodies used were horseradish peroxidase conjugates of anti-rabbit immunoglobulin G (Amersham Biosciences). Signals were detected by a Luminoimage Analyzer LAS-3000 (Fujifilm).

### 2.6. ELISA for determination of VEGF concentrations

The concentrations of VEGF in the culture supernatants were determined by an ELISA kit according to the manufacturer's instructions.

### 2.7. Transfection of HO-1 siRNA

Small interfering RNA for the rat HO-1 mRNA was transfected with the RNAiMAX lipofectaimine reagent (Invitrogen, Tokyo, Japan) and Opti-MEM medium (Invitrogen) according to the manufacture's protocols. The siRNA sequences of rat HO-1 mRNA were: 5′-GGAAUUUAUGCCAUGUAAATT-3′ and 5′-UUUACAUGGCA UAAAUUCCTT-3′.

### 2.8. Statistical analysis

A Dunnett's or Bonferroni's multiple comparison test, where appropriate, was applied to compare the data among the control and treated samples. $p$ Values less than 0.05 were regarded as statistically significant. These analyses were performed using GraphPad PRISM 4.0 (GraphPad Software, Inc., San Diego, USA).

## 3. Results

### 3.1. Sofalcone increases HO-1 expression in RGM-1 cells in time- and concentration-dependent manners

First we investigated the effect of sofalcone on the expression of HO-1 protein in RGM-1 cells. When RGM-1 cells were treated with sofalcone (50 μM) for 0–24 h, we observed the induction of HO-1 protein expression after 4 h treatment (Fig. 1A). The expression of HO-1 protein was strongly induced after 8 h and continued for 24 h. When the RGM-1 cells were treated with 20 μM or 50 μM of sofalcone for 24 h, the expression of HO-1 protein increased dose dependently (Fig. 1B). We also observed that 50 μM of sofal-

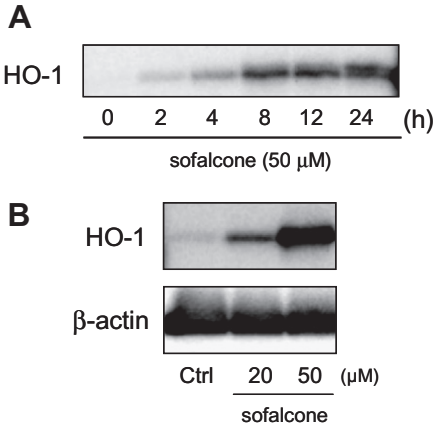


**Fig. 1.** Sofalcone increased HO-1 (32 kDa) protein expression in time- and concentration-dependent manners in RGM-1 cells. (A) 50 μM of sofalcone were treated for the various periods of time indicated, and the 32 kDa protein was detected with Western blotting. (B) RGM-1 cells were treated with 20 or 50 μM of sofalcone for 24 h. β-Actin (42 kDa) was used as an internal control.