Original Contribution

Willow bark extract increases antioxidant enzymes and reduces oxidative stress through activation of Nrf2 in vascular endothelial cells and *Caenorhabditis elegans*

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ARTICLE INFO

ABSTRACT

Willow bark extract (WBE) is listed in the European Pharmacopoeia and has been traditionally used for treating fever, pain, and inflammation. Recent studies have demonstrated its clinical usefulness. This study investigated the antioxidative effects of WBE in human umbilical vein endothelial cells (HUVECs) and *Caenorhabditis elegans*. WBE prevented oxidative-stress-induced cytotoxicity of HUVECs and death of *C. elegans*. WBE dose-dependently increased mRNA and protein expression levels of the nuclear factor erythroid 2-related factor 2 (Nrf2) target genes heme oxygenase-1, γ-glutamylcysteine ligase modifier and catalytic subunits, and p62 and intracellular glutathione (GSH) in HUVECs. In the nematode *C. elegans*, WBE increased the expression of the *gcs-1*::green fluorescent protein reporter, a well-characterized target of the Nrf2 ortholog SKN-1, in a manner that was SKN-1-dependent. WBE increased intranuclear expression and DNA binding of Nrf2 and the activity of an antioxidant response element (ARE) reporter plasmid in HUVECs. WBE-induced expression of Nrf2-regulated genes and increased GSH levels in HUVECs were reduced by Nrf2 and p38 small interfering (si) RNAs and by the p38-specific inhibitor SB203580. Nrf2 siRNA reduced the cytoprotective effect of WBE against oxidative stress in HUVECs. Salicin, a major anti-inflammatory ingredient of WBE, failed to activate ARE–luciferase activity, whereas a salicin-free WBE fraction showed intensive activity. WBE induced antioxidant enzymes and prevented oxidative stress through activation of Nrf2 independent of salicin, providing a new potential explanation for the clinical usefulness of WBE.

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Willow bark has been used as a traditional medicine for the treatment of fever, pain, and inflammation [1] and has proven effective in recent clinical trials in patients with osteoarthritis and low back pain [2,3]. The European Pharmacopoeia defines willow bark as the whole or fragmented dried bark of young branches or dried pieces of current-year twigs of various species of the genus *Salix*, including *Salix purpurea* L., *S. daphnoides* VILL., and *S. fragilis* L. [4]. Salicin, the major constituent of willow bark extract (WBE)[1], is metabolized to salicylic acid *in vivo* and has been considered to play a main role in its anti-inflammatory and analgesic effects. In addition, willow bark is specified to contain not less than 1.5% of total salicylic alcohol derivatives, expressed as salicin in the European Pharmacopoeia [4]. However, recent studies suggest that phenolic compounds other than salicin also contribute to the biological activities of WBE. Schmid et al. [5] demonstrated that the serum level of salicylate derivatives after the consumption of extract with 240 mg of salicin was insufficient to explain the analgesic or antirheumatic effects of willow bark. It has also been reported that phenolic compounds such as flavonoids and catechol isolated from WBE suppressed tumor necrosis factor (TNF)-α-induced inflammatory adhesion molecules in human microvascular endothelial cells [6]. Other studies have also suggested that compounds other than salicin contribute to the anti-inflammatory effects in lipopolysaccharide-activated

monocytes and to the inhibition of enzymes of arachidonic acid metabolism such as cyclooxygenase (COX)-1, COX-2, human leukocyte elastase, and 5-lipoxygenase [7,8]. However, the direct antioxidative properties of WBE containing many polyphenols have attracted attention as the explanation for its clinical application [9,10]. Further recent studies have also demonstrated that WBE reduced oxidative stress and increased glutathione (GSH) in several animal arthritis models [11,12]. These studies also suggested that WBE might suppress oxidative stress by inducing antioxidant enzymes, in addition to having radical-scavenging effects. However, the ability of WBE to induce antioxidant enzymes and the potential mechanisms have not been reported.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive master regulatory transcription factor that plays an important role in protecting against environmental oxidative stress [13–15]. Under normal conditions, Nrf2 is degraded when it is bound by the Kelch-like ECH-associated protein 1 (Keap1), a ubiquitin ligase subunit. Shear stress, dietary antioxidants, and other physiological stimuli that disrupt Keap1–Nrf2 interactions allow nuclear accumulation of Nrf2, resulting in the transcription of antioxidant and phase II defense enzymes such as heme oxygenase-1 (HO-1), γ-glutamylcysteine ligase (GCL), and NAD(P)H quinone oxidoreductase 1, through binding to the antioxidant response element (ARE) consensus sequence [16–18]. HO-1 is a rate-limiting enzyme in heme metabolism that has been recognized as an important protective factor in vascular tissue by exerting antioxidative, anti-inflammatory, antiproliferative, antiapoptotic, and vasodilatory effects on the vasculature [19]. HO-1 converts heme into vasculoprotective carbon monoxide and biliverdin, which is a potent antioxidant [20]. GCL is a heterodimer consisting of catalytic (GCLC) and modifier (GCLM) subunits, both of which are products of Nrf2 target genes. GCL has been extensively investigated for its ability to regulate the synthesis of GSH, which is the most abundant natural cellular antioxidant and plays an essential role in maintaining the cellular redox state [17,21]. Decreased intracellular GSH levels increase susceptibility to oxidative stress, and the resulting damage is thought to be involved in the onset and progression of various disease, including inflammatory, cardiovascular, metabolic, and neurodegenerative diseases [22,23]. It is therefore important to find ways to increase intracellular GSH levels to prevent oxidative stress-induced cellular damage.

In this study, we investigated the ability of WBE to prevent oxidative-stress-induced death of human umbilical vein endothelial cells (HUVECs) and to extend the longevity of Caenorhabditis elegans under conditions of oxidative stress. Furthermore, the effects of WBE on the expression of Nrf2-mediated antioxidant genes such as HO-1, GCLM, or GCLC and on increases in intracellular GSH were evaluated to elucidate the molecular mechanisms responsible for its preventive effects against oxidative stress.

Materials and methods

Reagents

MCDB 131 medium, L-glutamine, and peroxidase-linked anti-mouse antibody were purchased from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Biowest (Miami, FL, USA). Basic fibroblast growth factor was purchased from Kaken Pharmaceutical (Tokyo, Japan). WBE was obtained from Ask Intercity Co., Ltd. (Chiba, Japan). Tert-butylhydroperoxide (tBHP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), metaphosphoric acid, SB203580, salicin, and salicylic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-Nrf2 antibody (H-300), anti-lamin A/C antibody, and peroxidase-linked anti-rabbit antibody were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MAB374) was from Millipore (Billerica, MA, USA). Anti-HO-1 antibody was from Assay Design (Ann Arbor, MI, USA). Anti-GCLM antibody was purchased from Abcam (Cambridge, UK). Anti-p62 antibody was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Anti-p38 antibody, anti-phospho-p38 antibody, anti-phospho-ERK antibody, anti-phospho-JNK antibody, and anti-phospho-Akt antibody were from Cell Signaling (Danvers, MA, USA). Saligenin was purchased from MP Biomedicals (Solon, OH, USA).

Cell culture

HUVECs were cultured according to a previously reported method [24,25]. WBE was dissolved in serum-containing medium to the final desired concentration.

tBHP-induced oxidative stress assay in HUVECs

HUVECs at the fourth passage were seeded on 24-well type I collagen-coated plates. To determine the preventive effect of WBE on tBHP-induced cell toxicity, confluent cells were pretreated with 100 µg/ml WBE for 16 h, washed with phosphate-buffered saline (PBS), and exposed to tBHP (250 or 500 µM) for 6 h. Cell viability was determined by conventional MTT reduction assay. MTT is a tetrazolium salt cleaved to formazan by the mitochondrial respiratory chain enzyme succinate dehydrogenase. After treatment with tBHP, cells were incubated with MTT solution (0.5 mg/ml) in culture medium for 3 h. The culture medium was then removed, the formazan product was solubilized in dimethyl sulfoxide (DMSO), and the absorbance at 577 nm was measured using a microplate reader (Japan Intermed, NJ-2000). Values were expressed as percentage of cell survival. Absorbance from tBHP-untreated cells was set at 100%. To determine the involvement of Nrf2 in oxidative-stress-induced cell viability, cells were pretreated for 16 h with WBE 32 h after transfection with Nrf2 small interfering RNA (siRNA), and then exposed to tBHP for 6 h.

tBHP-induced oxidative stress assay in C. elegans

Stress resistance assay in C. elegans was performed according to previously reported methods [26–28]. Resistance of C. elegans to tBHP-induced oxidative stress was assessed in late L4 stage worms in nematode growth medium (NGM) plates containing WBE (10 mg/ml), fraction A (Fr. A; 5 µg/ml) or a control. The plates had been seeded with OP50 bacterial cultures. After incubation for 48 h at 20 °C, the worms were transferred with a small amount of bacteria to NGM plates containing freshly prepared 15.4 mM tBHP. In each experiment, two or three plates of 20 worms each were analyzed. Worms were scored as dead if they failed to respond to repeated gentle prodding with a platinum wire. Animals that crawled off the plate, ruptured, or died from internal hatching were excluded from analyses. All stress data were analyzed using JMP, with P values representing log rank. Table 1 shows the effects of WBE and Fr. A in the tBHP survival trial, related to Figs. 1B or 7C.

RNA extraction and real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the cells using a Total RNA mini kit (Bio-Rad, Hercules, CA, USA). Single-strand cDNA was synthesized from 0.5 µg of total RNA using a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). Quantitative analyses of HO-1, GCLM, GCLC, p62, Nrf2, and p38 mRNAs were performed by real-time RT-PCR using the ABI 7500 Fast Real-Time PCR system (Applied

Table 1

Effects of WBE and fraction A on tBHP survival trial in *Caenorhabditis elegans*.

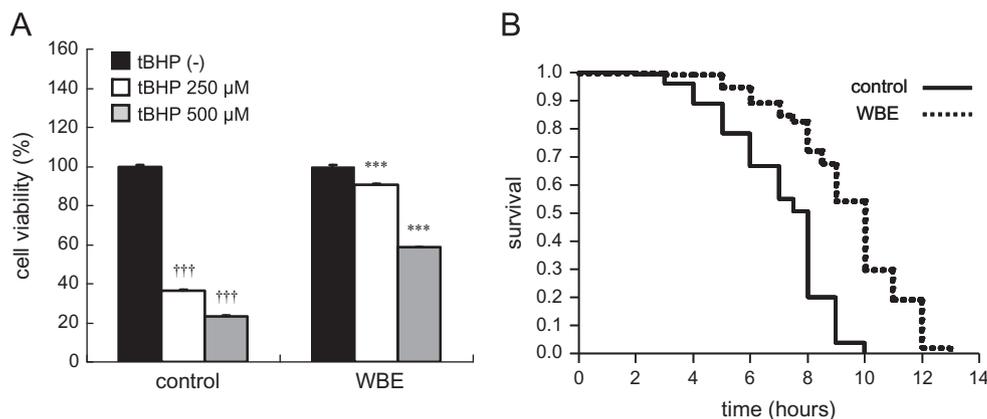| Test sample (+tBHP)	Mean survival (h ± SEM)	Median survival (h)	75th percentile (h)	P value (log-rank) vs control	% Mean survival extension	N	No. of experiments	Figure
Control	7.06 ± 0.2	8	8	–	–	126/145	3	1B
WBE	9.41 ± 0.1	10	8	< 0.0001	33	190/194	3	1B
Fr. A control	8.43 ± 0.2	9	10	–	–	95/105	2	7C
Fr. A	10.03 ± 0.1	10	11	< 0.0001	19	151/154	2	7C

Fig. 1. Preventive effects of WBE on tBHP-induced cytotoxicity in HUVECs. (A) HUVECs were pretreated with WBE (100 μg/ml) for 16 h and then stimulated with tBHP (250 and 500 μM) for 6 h. Cell viability was determined by MTT assay. Values represent the means ± SE of four experiments. ***$P < 0.001$, compared to tBHP-treated control, †††$P < 0.001$, compared to tBHP-untreated control. (B) Preventive effect of WBE on tBHP-induced death in *C. elegans*. N2 (wild-type) worms were exposed to WBE (10 mg/ml) for 48 h. Animals were then moved to plates containing 15.4 mM tBHP and scored every hour for movement and pharyngeal pumping. OP50 bacterial food was used as a control. The y axis indicates proportion surviving. See also Table 1 for statistics.

Biosystems, Foster City, CA, USA). Premix Ex Taq (Takara Bio) and Assay-on-Demand gene expression products (Hs01110250_m1 for HO-1, Hs00157694_m1 for GCLM, Hs00155249_m1 for GCLC, Hs00177654_m1 for p62, Hs00232352_m1 for Nrf2, Hs00176247_m1 for p38, and Hs02387368_g1 for RPS18; Applied Biosystems) were used for quantitative real-time RT-PCR analysis. Expression levels of mRNAs were quantified using standard curves. All quantitative data were normalized to the expression level of ribosomal protein S18 (RPS18).

Western blotting analysis

Whole-cell lysate from HUVECs was prepared in RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitor (Thermo Scientific) or phosphatase inhibitor (Thermo Scientific). Nuclear lysate was prepared using a nuclear/cytosol fractionation kit (BioVision, Mountain View, CA, USA), according to the manufacturer's protocol. Protein concentration was quantified using a BCA protein assay reagent kit (Thermo Scientific) using bovine serum albumin as a standard. Whole-cell and nuclear lysates were denatured by boiling in sodium dodecyl sulfate (SDS) sample buffer (Thermo Scientific), resolved by SDS–polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes by electroblotting. Blots were blocked and then incubated with rabbit anti-HO-1 primary antibody (1:10,000), rabbit anti-Nrf2 primary antibody (1:1000), rabbit anti-lamin A/C primary antibody (1:10,000) as an endogenous control, rabbit anti-phospho-p38 primary antibody (1:1000), rabbit anti-phospho-ERK primary antibody (1:1000), rabbit anti-phospho-JNK primary antibody (1:1000), rabbit anti-phospho-Akt primary antibody (1:1000), rabbit anti-p38 primary antibody (1:1000), mouse anti-GCLM primary antibody (1:1000), mouse anti-p62 primary antibody (1:1000), or mouse anti-GAPDH primary antibody (1:15,000) as an endogenous control overnight at 4 °C, followed by a horseradish peroxidase-linked secondary antibody for 1 h at room temperature,

and detected by chemiluminescence using an ImageQuant LAS 4000 mini system (GE Healthcare, Tokyo, Japan). Protein band intensities were quantified using ImageQuant TL (GE Healthcare).

Intracellular GSH measurement

Intracellular GSH levels were measured by washing cells twice with cold PBS and harvesting in 5% metaphosphoric acid using a cell scraper. Harvested cells were lysed by sonication on ice, and the supernatant of the centrifugate was collected. GSH levels were measured colorimetrically using a glutathione assay kit (GSH/GSSG-412; OxisResearch, Foster City, CA, USA) according to the manufacturer's protocol and normalized to the protein concentration in the lysate.

Transgenic analyses

Experiments were carried out in *C. elegans* using a *gcs-1* transgene fused to green fluorescent protein (GFP) (*gcs-1*::*GFP*), according to previous methods [26–29]. The premise was to subject *gcs-1*::*GFP* worms to WBE and observe the GFP expression levels in the intestines. The promoter mutant transgene *gcs-1*::*Δ2*::*GFP*, which lacks pharyngeal *gcs-1* expression, but maintains SKN-1-dependent expression in the ASI neurons and intestine, was also used, as well as *gcs-1*::*Δ2mut3*::*GFP*, which lacks a critical SKN-1 binding site in the *gcs-1*::*Δ2*::*GFP* promoter. For each experiment, approximately, 20 L4 stage worms were placed on fresh NGM plates containing OP50 bacteria and allowed to grow for 2–3 days, followed by treatment with WBE. The worms were transferred to an Eppendorf tube by flooding the plate with M9 saline medium and using a pipette to transfer them to the tube. After a quick spin, the M9 was removed and the animals were washed once more with M9 to remove any remaining bacteria. Once the worms were washed, they were incubated with WBE for the required time and then washed twice more in

M9, transferred to a fresh NGM plate containing bacteria, and allowed to recover for about 30 min. The worms were then mounted on slides and scored for GFP expression under the microscope. GFP expression in the intestine was scored as high, medium, or low, as described previously [27,29]. A high score was given if there was GFP expression throughout the intestine, a medium score for GFP expression midway up the intestine, and a low score for worms with little or no GFP expression in the intestine. For all transgenic reporter assays, the P values were determined by the χ^2 method.

Nrf2 DNA-binding assay

Nrf2 activation was assayed using Active Motif's (Carlsbad, CA, USA) enzyme-linked immunosorbent assay (ELISA)-based trans-activation TransAM kit, following the manufacturer's protocol. Nrf2 from nuclear lysate, which specifically binds to its consensus oligonucleotide, was analyzed colorimetrically using a spectro-photometer at 450 nm.

Cell transfection and luciferase assay

The ARE–luciferase reporter plasmid (pGL4.27(Nrf2-luc2P/minP/Hygro)) was obtained from Promega (Madison, WI, USA).

HUVECs (3.4×10^4) were plated in 12-well type I collagen-coated plates for 48 h. Cells were cotransfected with 0.2 µg luciferase expression plasmid and 0.05 µg of PRL-TK plasmid (Promega) as a normalization reference for transfection efficiency using Super-Fect transfection reagent (Qiagen, Valencia, CA, USA) for 8 h, and then the cells were stimulated with WBE, Frs. A–E, salicin, saligenin, or salicylic acid for 16 h. Cells were harvested, and firefly and *Renilla* luciferase activities were determined using a Dual-Luciferase Reporter assay system (Promega) with a lumin-ometer (GloMax 20/20n; Promega). The ratio (reporter/control luciferase activity) obtained from control cell lysate was set at 1.

Transfection with siRNA

HUVECs were plated in type I collagen-coated plates until 80–90% confluency. siRNA against Nrf2 or p38 was used to silence Nrf2 or p38, respectively (On-Target Plus SMARTpool reagent; Thermo Scientific). A control siRNA was also used (On-Target Plus Non-targeting siRNA 1; Thermo Scientific). HUVECs were transfected with 20 nM Nrf2 siRNA, p38 siRNA, or control siRNA using DharmaFECT 1 siRNA transfection reagent (Thermo Scientific) and incubated for 24 h in medium containing 2% FBS, after which the medium was refreshed. After incubation for a further 24 h, HUVECs were stimu-lated with WBE for 6 h to analyze the effects on mRNA expression.
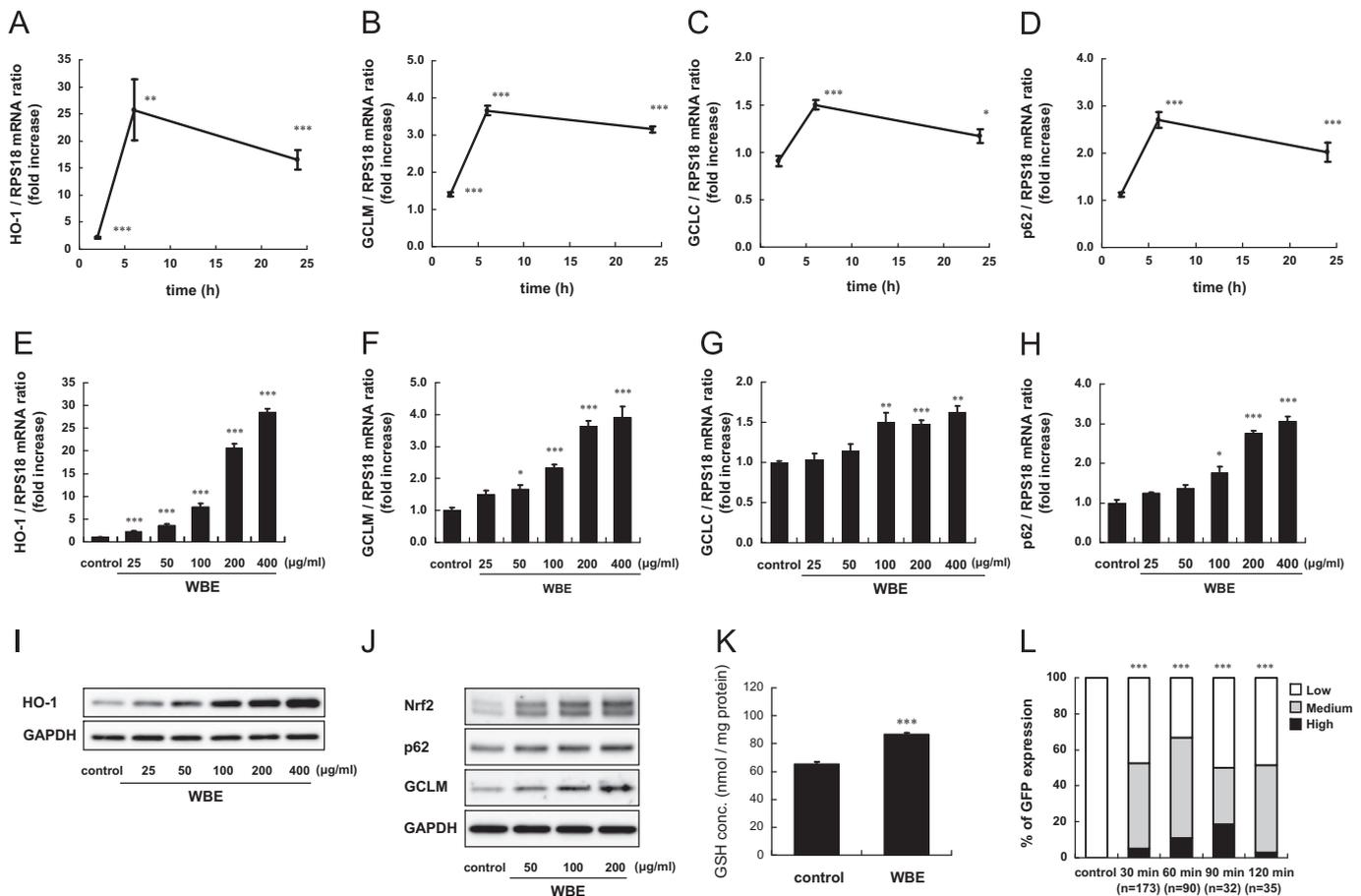

Fig. 2. Effects of WBE on HO-1, GCLM, GCLC, and p62 expression in HUVECs. (A–H) HUVECs were incubated with WBE (A–D, 200 µg/ml; E–H, 25–400 µg/ml) for 2–24 h. Relative mRNA expression levels were measured quantitatively using real-time RT-PCR. The results were normalized to RPS18 and expressed as fold increase over control. Values represent the means $\pm$ SE of four to six experiments. (I, J) HUVECs were incubated with WBE (25–400 µg/ml) for 16 h, and total cell lysates were subjected to Western blotting. (K) Effect of WBE on intracellular GSH content in HUVECs. HUVECs were incubated with WBE (200 µg/ml) for 16 h, and intracellular GSH content was determined. Values represent the means $\pm$ SE of six experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared to each corresponding control. (L) Effect of WBE on intestinal GFP expression of the *gcs-1::GFP* transgene in *C. elegans*. The indicated numbers of animals were exposed to WBE for 30–120 min and scored as having high, medium, or low intestinal GFP expression as described under Materials and methods. ***$P < 0.001$, compared to control.

To analyze protein expression, 32 h after transfection, cells were stimulated with WBE for 16 h and then subjected to Western blotting analyses. The silencing effects of Nrf2 and p38 were confirmed by real-time RT-PCR and Western blotting analyses.

Fractionation of WBE

WBE (50 g) was subjected to normal-phase silica gel (silica gel 60; Merck, Darmstadt, Germany) chromatography (CHCl$_3$:MeOH (10:1)→CHCl$_3$:MeOH:H$_2$O (7:3:1→6:4:1→5:5:1)→MeOH) to produce five fractions (A (5%), B (10%), C (21%), D (9%), E (55%)). The fractions were analyzed using high-performance liquid chromatography (HPLC), with a YMC-Pack ODS-AM (5 μm) 250 × 6 mm (YMC Co., Ltd., Kyoto, Japan) and a linear mobile-phase gradient combination of A, 0.2% acetic acid, and B, acetonitrile: gradient 0–95% B in 60 min, flow 2 ml/min. A diode array detector (190–400 nm) was used. The chromatograms shown in Fig. 7A were analyzed and plotted at 210 nm.

Statistical analysis

Data are presented as means ± SE. Differences between more than three groups were analyzed by two-tailed multiple *t* tests with Bonferroni correction. Comparisons between groups were analyzed using two-tailed Student *t* tests. Statistical significance was established at $P < 0.05$.

Results

Preventive effect of WBE on oxidative stress-induced toxicity in HUVECs and C. elegans

The inhibitory effects of WBE on oxidative stress-induced cellular damage were investigated in HUVECs pretreated with WBE, followed by induction of oxidative stress by tBHP, a lipid-soluble source of peroxide radicals. Cell viability was assessed by MTT assay 6 h after the induction of oxidative stress (Fig. 1A). Treatment with tBHP (250 and 500 μM) caused significant dose-dependent cytotoxicity, whereas pretreatment with WBE significantly protected against tBHP-induced cytotoxicity at a concentration of 100 μg/ml.

To investigate whether the protective WBE effect we observed might be evolutionarily conserved, we exposed C. elegans to oxidative stress by exposing them to tBHP, either with or without WBE treatment. The negative control for this group (OP50 bacteria alone) provided no protection against tBHP and all worms were dead at 10 h after exposure to tBHP. In contrast, some worms treated with WBE remained alive at 12 h (Fig. 1B).

WBE increases Nrf2-regulated antioxidant enzyme expression and intracellular GSH

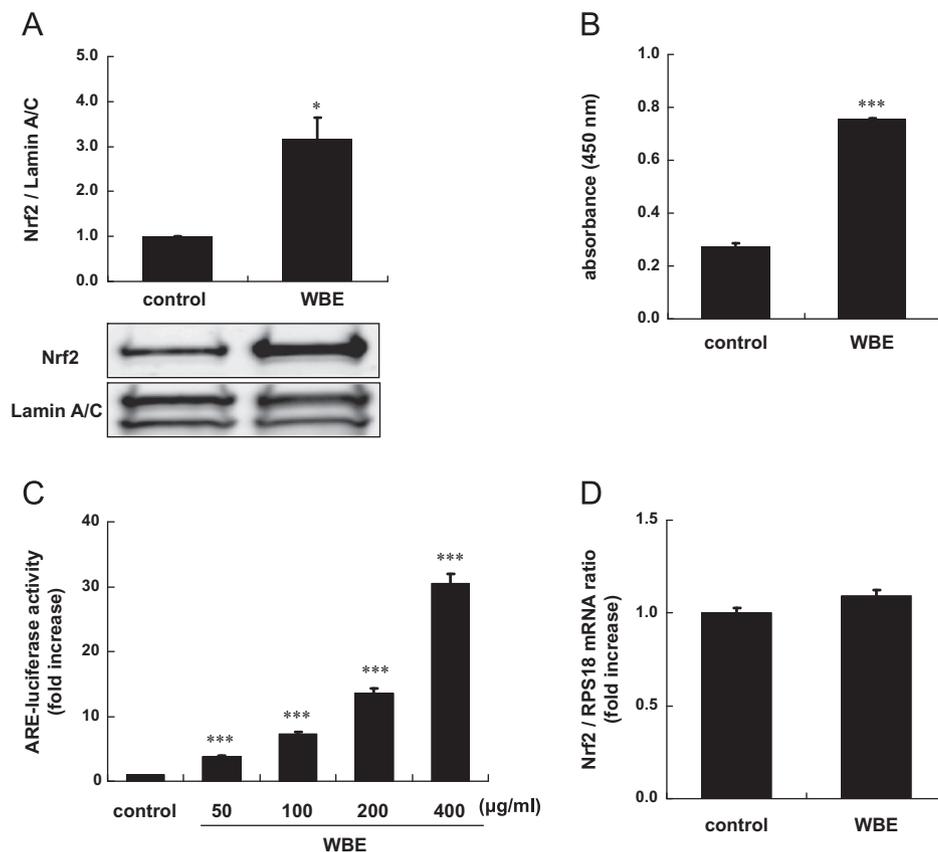To determine the effects of WBE on antioxidant enzymes, HUVECs were treated with 200 μg/ml WBE for 2, 6, or 24 h. WBE

Fig. 3. Effects of WBE on Nrf2 activation in HUVECs. (A) HUVECs were incubated with WBE (200 μg/ml) for 6 h. Nuclear lysates were subjected to Western blotting. Values represent the means ± SE of three experiments. (B) HUVECs were incubated with WBE (200 μg/ml) for 6 h. Analysis of the binding of Nrf2 in nuclear lysates to its consensus oligonucleotide was performed using the ELISA-based TransAM Nrf2 kit. Values represent the means ± SE of three experiments. (C) HUVECs were cotransfected with a reporter plasmid (pGL4.27(Nrf2-luc2P/minP/Hygro)) and a control plasmid (pRL-TK). After transfection, HUVECs were incubated with WBE (50–400 μg/ml) for 16 h, and then luciferase activity was determined. Values represent the means ± SE of three experiments. (D) HUVECs were incubated with WBE (200 μg/ml) for 6 h. Nrf2 mRNA expression was quantitated using real-time RT-PCR. The results were normalized to RPS18 and expressed as fold increase over control. Values represent the means ± SE of six experiments. *$P < 0.05$, ***$P < 0.001$, compared to each corresponding control.

caused maximal increases in HO-1, GCLM, and GCLC mRNA levels after 6 h (Figs. 2A–2C) and induced expression of another Nrf2 target gene, sequestosome 1 (p62), mRNA [30] in the same manner (Fig. 2D). Treatment with WBE at 25–400 μg/ml for 6 h dose-dependently increased their mRNA expression levels (Figs. 2E–2H). WBE also dose-dependently increased intracellular protein levels of HO-1, GCLM, and p62 (Figs. 2I and 2J) and significantly increased intracellular GSH (Fig. 2K) and intracellular Nrf2 in a dose-dependent manner (Fig. 2J).

The induction of an antioxidant gene by WBE in *C. elegans* was investigated by examining the promoter activity of *gcs-1* (GCLC homolog). *gcs-1* is known to encode a rate-limiting enzyme for glutathione synthesis and is a well-characterized target gene for SKN-1 (Nrf2 homolog) in *C. elegans*. Under normal conditions, *gcs-1*::GFP is expressed in the pharynx and ASI chemosensory neurons, but is induced in intestinal cells under oxidative stress conditions [29]. Treatment with 10 mg/ml WBE resulted in a dramatic increase in intestinal GFP expression compared to control conditions (Fig. 2L), with the maximal response at 60–90 min after treatment. Together, the data indicate that WBE increases expression of protective antioxidant enzymes that are regulated by Nrf2/SKN-1 proteins.

Activation of Nrf2 by WBE

We explored the role of transcription factor Nrf2 activation in mediating the increase in mRNA expression and promoter activity of antioxidant enzymes. Translocation of Nrf2 to the nucleus was evaluated by Western blotting of Nrf2 in the nuclear fraction of HUVECs. Treatment with WBE for 6 h increased Nrf2 in the nuclear lysates (Fig. 3A), and Nrf2 activation in HUVECs was induced by WBE (Figs. 3B and 3C). The binding activity of Nrf2 to its consensus oligonucleotide was significantly increased by stimulation with WBE, and ARE–luciferase activity was also increased in a dose-dependent manner. However, WBE had no effect on Nrf2 mRNA expression (Fig. 3D).

Antioxidant gene induction by WBE is dependent on Nrf2/SKN-1

The role of Nrf2 in the induction of HO-1, GCLM, GCLC, and p62 by WBE was investigated by transfecting HUVECs with Nrf2 siRNA to decrease Nrf2 expression, using scrambled siRNA as a control. Nrf2 was downregulated after treatment with Nrf2 siRNA, as confirmed by real-time RT-PCR and Western blotting analyses.
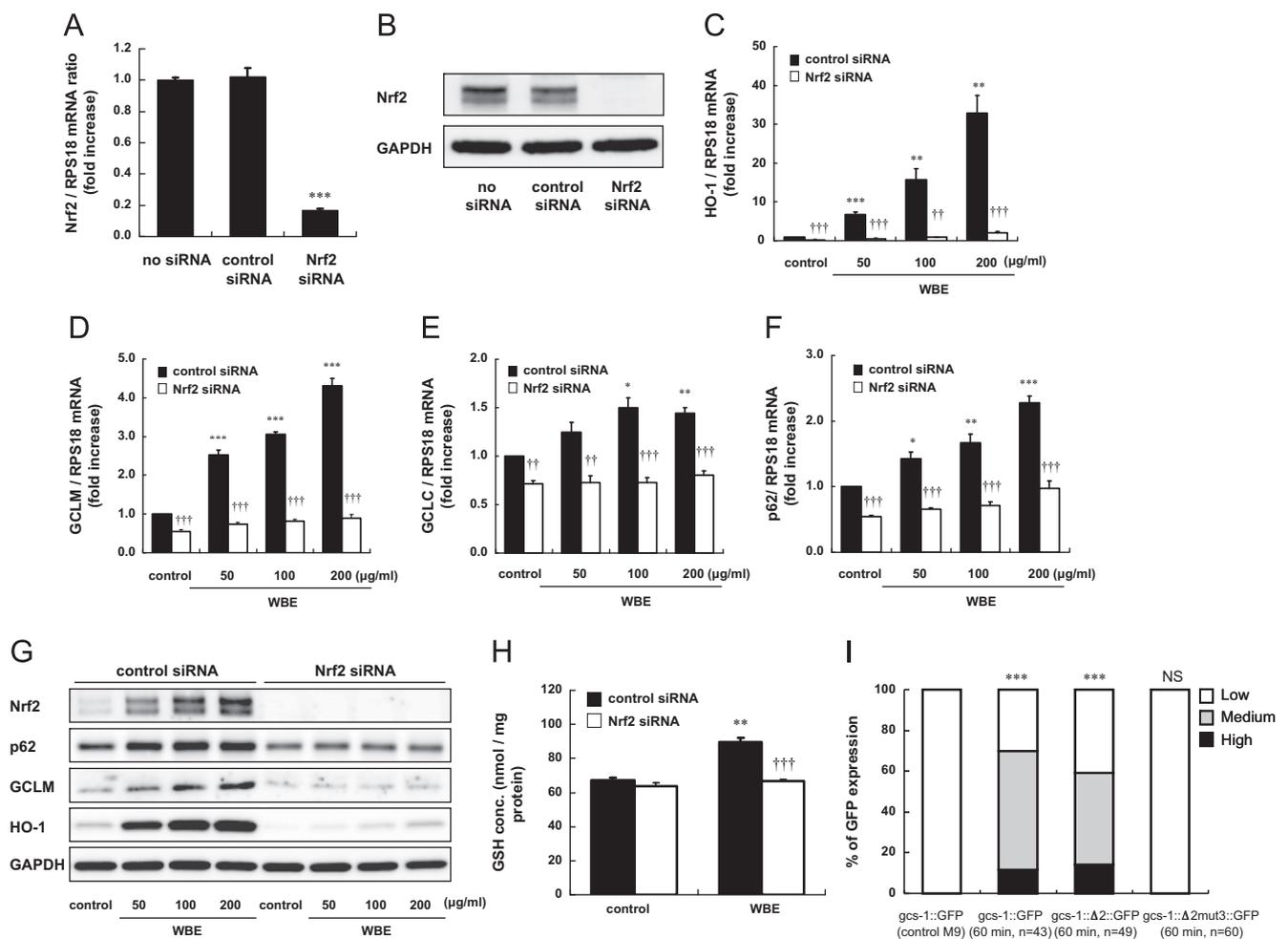

Fig. 4. Effects of WBE on the expression of HO-1, GCLM, GCLC, and p62 in Nrf2 knockdown HUVECs. (A, B) HUVECs were treated with Nrf2 siRNA or control siRNA and incubated for 48 h. (A) Nrf2 mRNA expression was quantitated and normalized as for Fig. 3D. Values are expressed as the means ± SE of four experiments. (B) Whole-cell lysates were subjected to Western blotting. (C–F) HUVECs were transfected with siRNA against Nrf2 or control siRNA. After 48 h, the cells were incubated with WBE (50–200 μg/ml) for an additional 6 h. Relative mRNA expression levels were analyzed using real-time RT-PCR. Values are expressed as the means ± SE of four experiments. (G) HUVECs were transfected with siRNA against Nrf2 or control siRNA. After 32 h, the cells were incubated with WBE (50–200 μg/ml) for a further 16 h. Whole-cell lysates were subjected to Western blotting. (H) Effect of WBE on intracellular GSH content in Nrf2 knockdown HUVECs. WBE (200 μg/ml) was applied in a manner similar to that for (G), and intracellular GSH content was determined. Values represent the means ± SE of three experiments. *P < 0.05, **P < 0.01, ***P < 0.001, compared to control cells treated with control siRNA; ††P < 0.01, †††P < 0.001, compared to the corresponding cells treated with control siRNA. (I) Effect of WBE on intestinal GFP expression in *C. elegans* with *gcs-1*::Δ2::GFP or *gcs-1*::Δ2mut3::GFP transgene. The indicated numbers of animals with each transgene were exposed to WBE (10 mg/ml) for 60 min and scored as high, medium, or low expressing based on the levels of GFP expression in the intestine. ***P < 0.001, compared to *C. elegans* with *gcs-1*::GFP.

The expression of Nrf2 mRNA in cells treated with Nrf2 siRNA was reduced by approximately 80% (Fig. 4A), and Nrf2 protein expression in whole-cell lysate was also markedly suppressed (Fig. 4B). The increases in HO-1, GCLM, GCLC, and p62 mRNA expression levels caused by WBE (50–200 μg/ml) were significantly suppressed by Nrf2 siRNA (Figs. 4C–4F). Similarly, knockdown of Nrf2 also reduced WBE-induced HO-1, GCLM, and p62 protein expression levels (Fig. 4G). Furthermore, the increase in intracellular GSH induced by WBE was significantly reduced by knockdown of Nrf2 (Fig. 4H).

gcs-1::Δ2::GFP animals were used to test the dependence of the effects of WBE on SKN-1 [29]. This promoter mutant transgene lacks SKN-1-independent pharyngeal *gcs-1* expression, but maintains SKN-1-dependent expression in the ASI neurons and intestine. *gcs-1::Δ2::GFP* worms treated with WBE displayed the same expression patterns as *gcs-1::GFP* worms treated with WBE for 60 min (Fig. 4I). The mutant transgene *gcs-1::Δ2mut3::GFP* was used to determine if induction by WBE required SKN-1. This variant of *gcs-1::Δ2::GFP* lacks a critical SKN-1 binding site in its promoter and shows no GFP expression in the pharynx, ASI neurons, or intestine under normal and stress conditions [29]. *C. elegans* transfected with this mutant gene and treated with WBE lacked GFP expression, with expression in 100% of worms scored as low (Fig. 4I). We conclude that WBE promotes antioxidant gene expression by acting through Nrf2/SKN-1 proteins.

Cytoprotection against oxidative stress by WBE is dependent on Nrf2

The role of Nrf2 in the cytoprotective effect of WBE against tBHP-induced cell toxicity was investigated in Nrf2 knockdown HUVECs. WBE suppressed tBHP (250 or 500 μM)-induced cell toxicity in cells treated with control siRNA, and the protective effects were significantly reduced in Nrf2 knockdown cells (Fig. 5), indicating that this cytoprotection was mediated through the action of Nrf2.

WBE-induced HO-1 and GCLM expression levels in HUVECs are dependent on p38

The molecular mechanisms whereby WBE activated Nrf2 were investigated by assessing its impact on phosphorylation of p38,
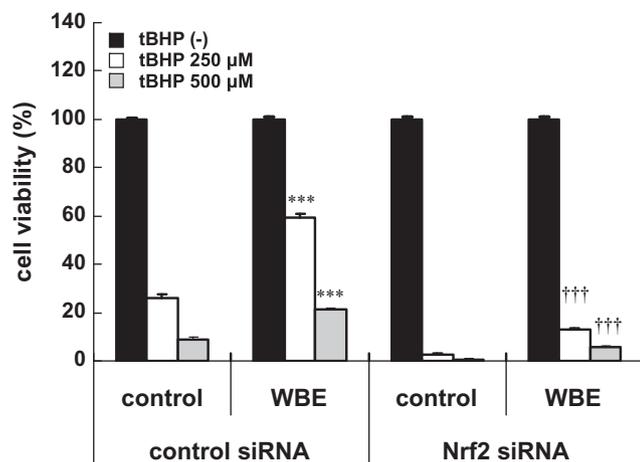
Fig. 5. Effects of WBE on tBHP-induced cytotoxicity in Nrf2 knockdown HUVECs. HUVECs were transfected with siRNA against Nrf2 or control siRNA. After 32 h, the cells were incubated with WBE (100 μg/ml) for a further 16 h and then stimulated with tBHP (250 and 500 μM) for 6 h. Cell viability was determined by MTT assay. Values represent the means ± SE of eight experiments. ***$P < 0.001$, compared to tBHP-treated control transfected with control siRNA; †††$P < 0.001$, compared to tBHP-treated WBE transfected with control siRNA.

ERK, JNK, and Akt for up to 6 h. The phosphorylation of p38 was detected between 30 and 180 min, in contrast to its effects on ERK, JNK, and Akt (Fig. 6A). HUVECs were therefore treated with the specific p38 inhibitor SB203580, or with p38 siRNA, and the HO-1 and GCLM mRNA and protein expression levels were evaluated to elucidate the role of p38 in mediating Nrf2 activation. SB203580 showed a statistically significant but not complete inhibition of WBE-induced HO-1 and GCLM mRNA expression (Figs. 6B and 6C). The expression of p38 mRNA in cells treated with p38 siRNA was reduced by approximately 80% (Fig. 6D), and p38 protein expression in whole-cell lysate was also markedly suppressed (Fig. 6E). WBE-induced increases in HO-1 and GCLM mRNA expression levels were also significantly reduced in p38 knockdown cells (Figs. 6F and 6G). Similarly, HO-1, GCLM, and Nrf2 proteins in whole-cell lysate were increased by WBE, but were clearly reduced in HUVECs transfected with p38 siRNA (Fig. 6H).

Effects of WBE fractions and salicin on ARE–luciferase activity in HUVECs

To determine the contribution of salicin to the Nrf2-mediated antioxidative activity of WBE, WBE was separated into five fractions (Frs. A–E), and their effects on ARE–luciferase activity were investigated, together with those of salicin, saligenin, and salicylic acid, as metabolites of salicin. HPLC patterns for WBE, Frs. A–E, and salicin are shown in Fig. 7A. The major peak in the salicin standard chromatogram was confirmed at 15.1 min. Salicin was also confirmed to be rich in WBE and was especially concentrated in Fr. C, whereas Fr. A contained no salicin. The ARE–luciferase activities of Frs. A–E, salicin, saligenin, and salicylic acid are shown in Fig. 7B. WBE (50 μg/ml) showed similar ARE–luciferase activity compared to Fig. 3C. Fractions A and B showed more intensive activities than Frs. C–E at a concentration of 50 μg/ml, whereas salicin and its metabolites were incapable of stimulating any activity.

Preventive effect of Fr. A on oxidative-stress-induced death of C. elegans

The antioxidative effect of Fr. A, which showed the greatest ability to activate Nrf2, was tested in *C. elegans* treated with 5 μg/ml Fr. A and then exposed to oxidative stress with tBHP. DMSO was used as a negative control. Animals treated with Fr. A (5 μg/ml) were significantly resistant to tBHP-induced death, similar to the effect of WBE (Fig. 1B), at a lower concentration (Fig. 7C). Together, the data indicate that WBE contains salicin-independent activities that promote antioxidant enzyme expression and stress resistance by acting on Nrf2/SKN-1.

Discussion

Recent studies have shown that WBE increases GSH levels as well as reducing inflammatory mediators, resulting in inhibition of lipid peroxidation in several animal arthritis models [11,12]. These and other studies have proposed a direct radical-scavenging activity of the polyphenols to explain the protective effects of WBE against oxidative stress [9,10]. However, it has not been determined whether WBE might act indirectly through Nrf2 or other pathways. The results of this study demonstrated that WBE stimulated the expression of antioxidant enzyme genes, including HO-1, GCLM, and GCLC, and increased intracellular GSH, leading to its protective effects against oxidative damage in HUVECs, as well as in *C. elegans*. WBE also increased ARE–luciferase activity and Nrf2 translocation to the nucleus in HUVECs and stimulated *gcs-1* promoter activity in an SKN-1 site-dependent manner in *C. elegans*. Knockdown of Nrf2 with siRNA inhibited not only
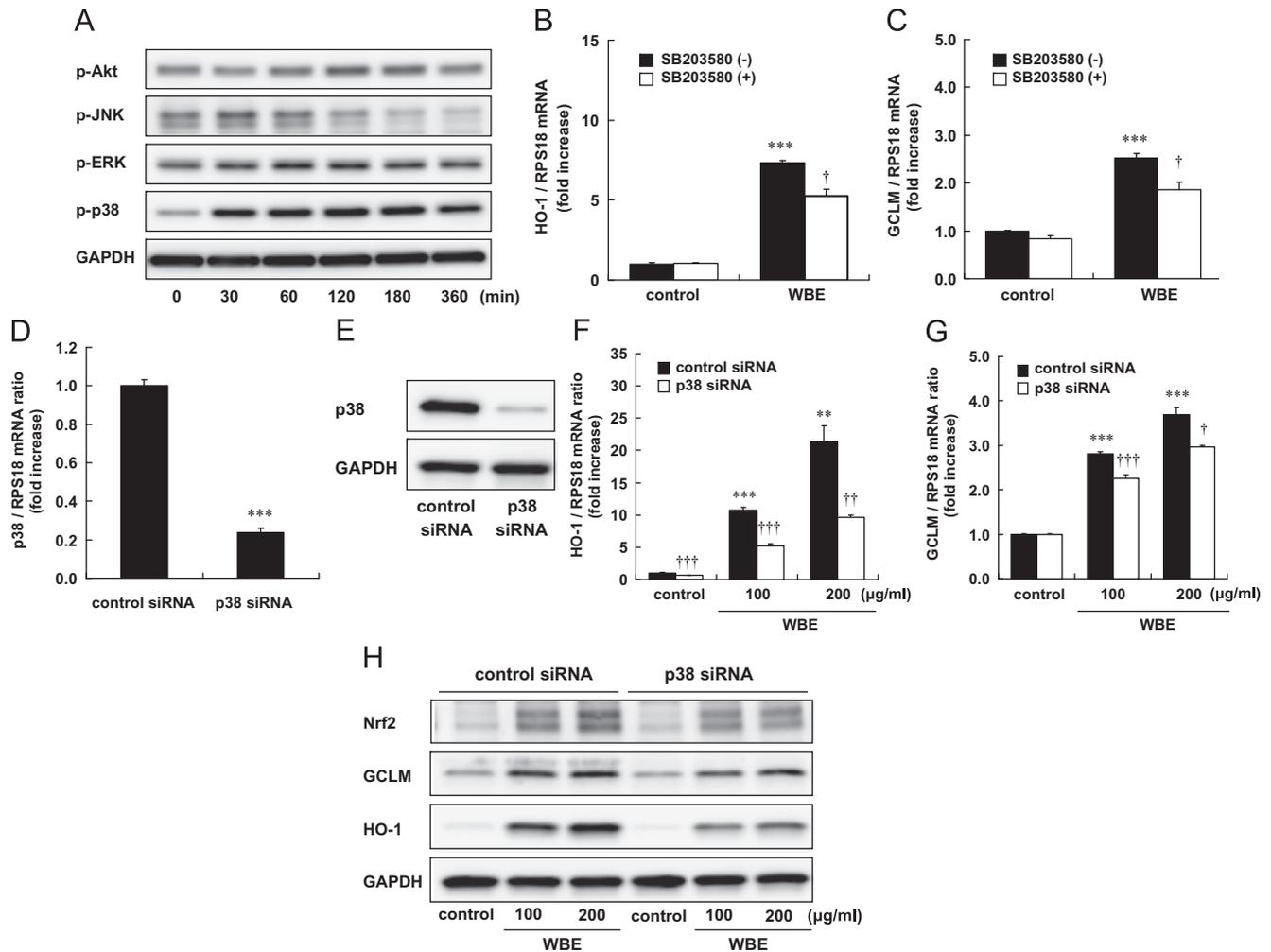
Fig. 6. Effects of WBE on phosphorylation of p38, ERK, JNK, and Akt in HUVECs. (A) HUVECs were incubated with WBE (400 µg/ml) for 0–360 min. Total cell lysates were subjected to Western blotting. (B, C) Effects of SB203580 on WBE-induced HO-1 and GCLM mRNA expression, quantitated by real-time RT-PCR and normalized as for Fig. 3D. HUVECs were pretreated with SB203580 (20 µM) for 1 h and treated with WBE (100 µg/ml) for an additional 6 h. Values are expressed as the means ± SE of three experiments. ***$P < 0.001$, compared to the SB203580-untreated control; †$P < 0.05$, compared to SB203580-untreated WBE. (D–H) Effects of WBE on the expression levels of HO-1 and GCLM in p38 knockdown HUVECs. (D) HUVECs were treated with p38 siRNA or control siRNA and incubated for 48 h. The relative mRNA expression of p38 was quantitated using real-time RT-PCR. Values are expressed as the means ± SE of six experiments. (E) Whole-cell lysates were subjected to Western blotting. (F, G) HUVECs were transfected with siRNA against p38 or control siRNA. After 48 h, the cells were incubated with WBE (100–200 µg/ml) for a further 6 h. The relative expression of mRNAs was quantitated using real-time RT-PCR. Values are expressed as the means ± SE of three to six experiments. (H) HUVECs were transfected with siRNA against p38 or control siRNA. After 32 h, the cells were incubated with WBE (100–200 µg/ml) for a further 16 h. Whole-cell lysates were subjected to Western blotting. **$P < 0.01$, ***$P < 0.001$, compared to control cells treated with control siRNA; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, compared to the corresponding cells treated with control siRNA.

WBE-induced expression of these antioxidant enzymes, but also the protective effect against oxidative stress-induced cellular damage in HUVECs. This study is the first to demonstrate that WBE increases antioxidant enzymes and prevents oxidative-stress-induced cell death through activation of Nrf2. Recent studies have reported that activation of Nrf2 suppresses inflammatory mediators including TNF-α, monocyte chemoattractant protein-1, and vascular cell adhesion molecule-1 in vascular endothelial cells and macrophages [31–34]. Furthermore, several studies have shown that Nrf2 could be a protective targeting molecule for the treatment of arthritis [35,36]. These results suggest that the activation of Nrf2 by WBE might contribute to its clinical effects in inflammatory disorders.

The molecular mechanisms responsible for Nrf2 activation by WBE were also evaluated in this study. A recent study identified p62 as a target gene of Nrf2 [30] and found that it interacted with the Nrf2-binding sites on Keap1. Overproduction of p62 competes with the interaction between Nrf2 and Keap1, resulting in stabilization of Nrf2 and transcriptional activation of Nrf2 target genes [37]. It has also been suggested that JNK activation-induced

p62 expression is required for Nrf2-induced GSH synthesis in cultured human retinal pigment epithelial cells [38]. We therefore investigated the role of p62 in the induction of Nrf2 target genes using siRNA targeted against p62. Treatment of HUVECs with p62 siRNA markedly downregulated p62 expression, but did not change the expression of Nrf2 target genes (data not shown), indicating that the WBE-induced p62 expression observed in this study depended entirely on Nrf2 activation. Phosphorylation of Nrf2 is also known to play an important role in the regulation of Nrf2 activation, and many studies have revealed that signaling mediated through protein kinase C, phosphoinositide 3-kinase–Akt, or mitogen-activated protein kinases (MAPKs) such as p38, ERK, and JNK is involved in the activation of Nrf2 in several cell types [39–45]. In this study, the phosphorylation of p38 was clearly increased by the treatment with WBE, in contrast to other MAPKs or Akt in HUVECs. The p38-specific inhibitor SB203580 and siRNA targeted against p38 significantly but not completely inhibited WBE-induced increases in expression of the Nrf2 target genes HO-1 and GCLM, as well as intracellular increases in Nrf2 protein, suggesting that p38 signaling was partially involved in
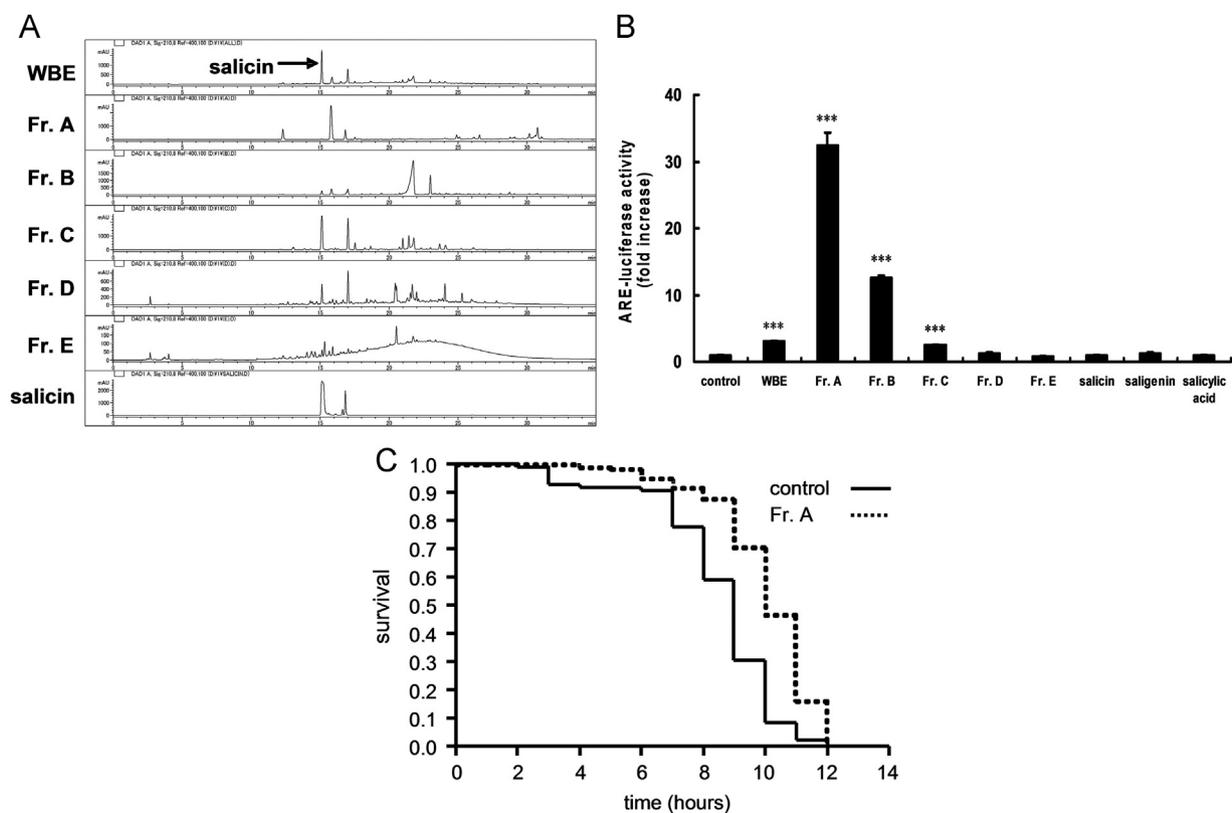
Fig. 7. HPLC chromatograms of WBE, Frs. A–E, and salicin. (A) WBE, Frs. A–E, and salicin were analyzed by HPLC using an ODS column and plotted at 210 nm. (B) Effects of Frs. A–E, salicin, saligenin, and salicylic acid on ARE–luciferase activity in HUVECs. HUVECs were cotransfected with a reporter plasmid (pGL4.27(Nrf2-luc2P/minP/Hygro)) and a control plasmid (pRL-TK). After transfection, the HUVECs were incubated with WBE (50 µg/ml), Frs. A–E (50 µg/ml each), salicin (100 µM), saligenin (100 µM), or salicylic acid (100 µM) for 16 h, and then luciferase activity was determined. Values represent the means ± SE of three experiments. ***$P < 0.001$, compared to control. (C) Preventive effect of Fr. A on tBHP-induced death in *C. elegans*. N2 worms were exposed to Fr. A (5 µg/ml) for 48 h on plates. Animals were then moved to plates containing 15.4 mM tBHP and scored every hour for movement and pharyngeal pumping. 0.06% DMSO-containing M9 was used as a control. The *y* axis indicates proportion surviving. See also Table 1 for statistics.

Nrf2 activation by WBE. In fact, in *C. elegans* it has been shown that p38 phosphorylates SKN-1 directly in response to oxidative stress, leading to its accumulation in intestinal nuclei [46]. Therefore, WBE seems to act on a step upstream of this evolutionarily conserved mechanism. However, further studies are also needed to elucidate the precise mechanism involved.

Salicin is a major active component of WBE, and the WBE used in this study contained more than 15% salicin. We therefore investigated whether the activation of Nrf2 by WBE was attributable to salicin, saligenin, or salicylic acid, as metabolites of salicin. However, these compounds failed to activate Nrf2 as assessed by ARE–luciferase activity in HUVECs. We fractionated WBE using normal-phase silica gel chromatography and examined the effects of each fraction on ARE–luciferase activity in HUVECs. Fractions A and B, which contained little salicin as the less hydrophilic fractions, showed more intense activity than the salicin-containing fractions (Frs. C and D). Furthermore, Fr. A extended the survival of *C. elegans* exposed to oxidative stress with tBHP. These results clearly showed that active ingredients in WBE, other than salicin, contributed significantly to the Nrf2-mediated increase in antioxidant enzymes and prevention of oxidative stress, though further studies are needed to identify the active ingredient.

Conclusions

In conclusion, this study demonstrated that WBE simulates the expression of antioxidant enzymes and prevents oxidative stress through activation of Nrf2 in vascular endothelial cells and *C. elegans*, providing a novel explanation for its clinical usefulness. Moreover, p38 signaling is partially involved in the effects of WBE, and compounds other than salicin may contribute to the effects of WBE on Nrf2 activation. SKN-1/Nrf2 signaling has been linked to longevity in *C. elegans*, *Drosophila*, and mice, and Nrf2 activation has attracted attention as a target molecule for various diseases, including inflammatory diseases [27,47–49]. Therefore, WBE might have broad applicability in the setting of chronic and aging-related disease in addition to acute stress, and identification of the WBE constituent responsible for the activation of Nrf2 represents a potentially new approach for the development of Nrf2-related therapeutic agents.

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