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Original Research Communication

Fragrant Unsaturated Aldehydes Elicit Activation of the Keap1/Nrf2 System Leading to the Upregulation of Thioredoxin Expression and Protection Against Oxidative Stress

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Abstract

Thioredoxin, a key molecule in redox regulation, and many redox enzymes are regulated through the antioxidant responsive element (ARE). To search for antioxidative constituents, we screened extracts from vegetables and found that the extracts of *Perilla frutescens* and *Artemisia princeps* have potent thioredoxin-inducing activities. By activity-guided purification of *Perilla frutescens* extracts, we identified perillaldehyde as a novel thioredoxin inducer. Fragrant unsaturated aldehydes, such as *trans*-cinnamaldehyde, safranal, 2,4-octadienal, citral, *trans*-2, *cis*-6-nonadienal, and *trans*-2-hexenal showed the ability to activate ARE. Perillaldehyde-induced activation through the ARE was suppressed by the overexpression of wild-type Keap1, whereas sulforaphane-induced activation seemed to be partially suppressed. Mutant Keap1 (R272A/K287A or C273A/C288A) did not suppress this activation. Pretreatment with perillaldehyde reduced the H_2O_2-induced cytotoxicity. Thus, we show that fragrant unsaturated aldehydes from edible plants are novel thioredoxin inducers and ARE activators and may be beneficial for protection against oxidative stress–induced cellular damage. These results also suggest that perillaldehyde activates the Nrf2-Keap1 system and that the lysine and arginine residues juxtaposed to the critical cysteine residues of Keap1 are required for signal sensing. *Antioxid. Redox Signal.* 11, 949–962.

Introduction

Thioredoxin was identified in *Escherichia coli* as an electron donor for ribonucleotide reductase (18) and acts as a key molecule for redox regulation by catalyzing protein disulfide reductions in combination with thioredoxin reductase and NADPH (9). Human thioredoxin was cloned from human T-cell leukemia virus type I–infected cells (31). Thioredoxin regulates cellular processes *via* reduction/oxidation (redox regulation) (22) and serves as a hydrogen donor for families of thioredoxin-dependent peroxidases (peroxiredoxin) (5, 29). Homozygous mutants carrying a targeted disruption of the thioredoxin gene died shortly after implantation, suggesting that thioredoxin expression is essential for cell survival and early differentiation in the mouse embryo (23). Transgenic mice expressing human thioredoxin display various phenotypes, such as an elongated life span and protection against ischemic injury, acute lung failure, diabetes mellitus, thioacetamide-induced liver injury, and toxicity caused by environmental stressors (11, 12, 24, 27, 32, 39). Because oxidative stress has been implicated in the pathogenesis of these diseases, thioredoxin seems to play an important role in protection against oxidative stress–associated diseases. The identification of thioredoxin inducers may provide a preventive approach against such oxidative stress–associated diseases.

The thioredoxin gene is induced by nerve growth factor through the cyclic AMP–responsive element in PC12 cells (1) and by hemin, *tert*-butylhydroquinone, or sulforaphane through the antioxidant-responsive element (ARE) in K562

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Table 1. Plants of Which Extracts are Examined in this Study

Mustard family	***Brassicaceae***
Broccoli	*Brassica oleracea var. italica*
Cabbage	*Brassica oleracea var. capitata*
Red cabbage	*Brassica oleracea var. capitata*
Savoy cabbage	*Brassica oleracea var. capitata*
Brussels sprouts	*Brassica oleracea var. gemmifera*
Kale	*Brassica oleracea var. acephala*
Daikon radish	*Raphanus sativus*
Turnip	*Brassica rapa*
Radish	*Raphanus sativus var. radicula*
Rocket	*Eruca vesicaria*
Watercress	*Nasturtium officinale*
Chinese cabbage	*Brassica rapa L. var. gabra Regel*
Cauliflower	*Brassica oleracea var. botrytis*
Pot herb mustard	*Brassica rapa var. nipposinica*
Oriental mustard	*Brassica juncea*
Qing geng cai	*Brassica rapa var. chinensis*
Chinese flat cabbage	*Brassica chinensis var. rosularis*
Japanese horse-radish	*Eutrema japonica*
Mibu-na	*Brassica campestris var. laciniifolia*
Shirona	*Brassica campestris var. amplexicaulis*
Potato family	***Solanaceae***
Chilli pepper (Fushimi, green and red)	*Capsicum annuum*
Chilli pepper (Manganji, green and red)	*Capsicum annuum*
Chilli pepper (Takagamine)	*Capsicum annuum*
Jalapeno	*Capsicum annuum*
Paprika (green and red)	*Capsicum annuum cv.*
Chilli pepper	*Capsicum annuum*
Eggplant (Kamo)	*Solanum melongena*
Eggplant (Yamanashi)	*Solanum melongena*
Potato	*Solanum tuberosum*
Potato (purple)	*Solanum tuberosum*
Potato (yellow)	*Solanum tuberosum*
Tomato	*Solanum lycopersicum*
Carrot family	***Apicaceae***
Carrot	*Daucus carota*
Carrot (Shima)	*Daucus carota var. satira DC.*
Carrot (Kintoki)	*Daucus carota*
Japanese parsley	*Oenanthe javanica*
Japanese honeywort	*Cryptotaenia japonica Hassk.*
Parsley	*Petroselium crispum*
Sweet fennel	*Foeniculum vulgare var. dulce*
Ashitaba	*Angelica keiskei*
Mint family	***Lamiaceae***
Perilla herb (Aka-Shiso)	*Perilla frutescens var. acuta*
Perilla herb (Ao-Shiso)	*Perilla frutescens var. acuta*
Lily family	***Liliaceae***
Onion	*Allium cepa*
Red onion	*Allium cepa*
Shallot	*Allium ascalonicum*
Scallion (Kujyo)	*Allium fistulosum*
Chive	*Allium schoenoprasum var. foliosum*
Asparagus	*Asparagus officinalis*
Scallion	*Allium fistulosum*
Leek	*Allium ampeloprasum var. porrum*
	Zingiberaceae
Ginger	*Zingiber officinale*
Turmeric	*Curcuma longa*
Turmeric	*Curcuma aromatica*
Aster family	***Asteraceae***
Mugwort	*Artemisia princeps*
Chop-suey greens	*Chrysanthemum coronarium*
Chicory	*Cichorium intybus*
Hosobawadan	*Crepidiastrum lanceolatum Nakai*
Suizenji-na	*Gynura bicolor*
Gourd family	***Cucurbitaceae***
Cucumber	*Cucumis sativus*
Celery	*Apium graveolens var. dulce*
Pumpkin (Shishigatani)	*Cucurtita moschata*
Bitter melon	*Momordica charantia var. pavel Crantz*
	Chenopodiaceae
Spinach	*Spinacia oleracea*
Red beet	*Bet vulgaris ssp.hortensis*
Pea family	***Fabaceae***
Soybean (Kuromame)	*Glycine max*
Soybean	*Glycine max*
Winged bean	*Psophocarpus tetragonolobus*
	Polygonaceae
Buckwheat	*Fagopyrum esculentum*
Water pepper	*Polygonum hydropiper*
Others	
Taro	*Colocasia esculenta*
Kuwa	*Morus bombycis*
Mulukhiyya	*Corchorus olitorius*
Papaya	*Carica papaya*
Aloe	*Aloe vera*
Myoga	*Zingiber mioga*
Yacon	*Polymnia sonchifolia*

erythroleukemia cells (14, 15, 21) and retinal pigment epithelial cells (34). The expression of genes encoding redox-regulating enzymes, such as γ-glutamylcysteine synthetase (7), glutathione *S*-transferase (GST) Ya, NAD(P)H/quinone oxidoreductase, peroxiredoxin, thioredoxin reductase (8), and hemeoxygenase-1 is regulated through AREs (26) and the transcription factor Nrf2 (13). A wide variety of substances are reported to activate Nrf2 through ARE. Among them, isothiocyanates, such as sulforaphane, have been most intensively studied (4). Electrophilic substances are considered to interact with Keap1, changing the conformation and the ubiquitination activity of Keap1 to stabilize Nrf2 protein (16). Cysteine residues 273 and 288 of Keap1 have been shown to be essential for Keap1 to repress Nrf2 activity *in vivo* (38). However, the mechanism underlying the activation of Keap1 by various types of stimuli remains to be elucidated.

Thioredoxin-inducing and ARE-activating constituents in Japanese vegetables, other than Brassicaceae plants, and the mechanisms underlying this activation, have not been thoroughly investigated. In addition, although a chemopreventive effect of sulforaphane has been reported, some derivatives of isothiocyanates have cytotoxic activities. This background prompted us to screen extracts from commonly edible Japanese vegetables for novel thioredoxin inducers. Here we show potent thioredoxin-inducing and ARE-activating activity in the extracts of *Perilla frutescens* and *Artemisia princeps* and demonstrate that perillaldehyde from *P. frutescens* and other fragrance-unsaturated aldehydes induce the expression of the

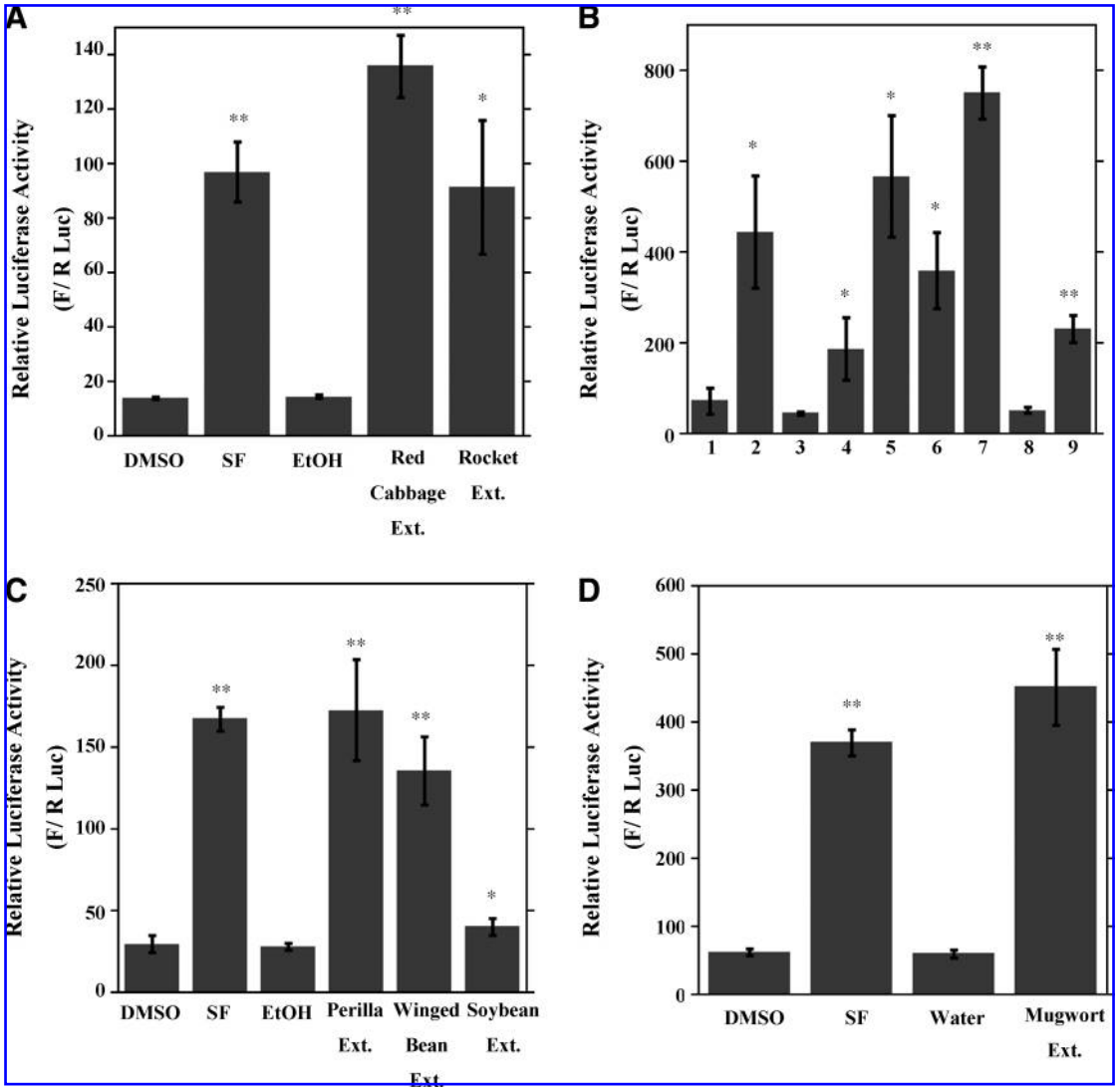


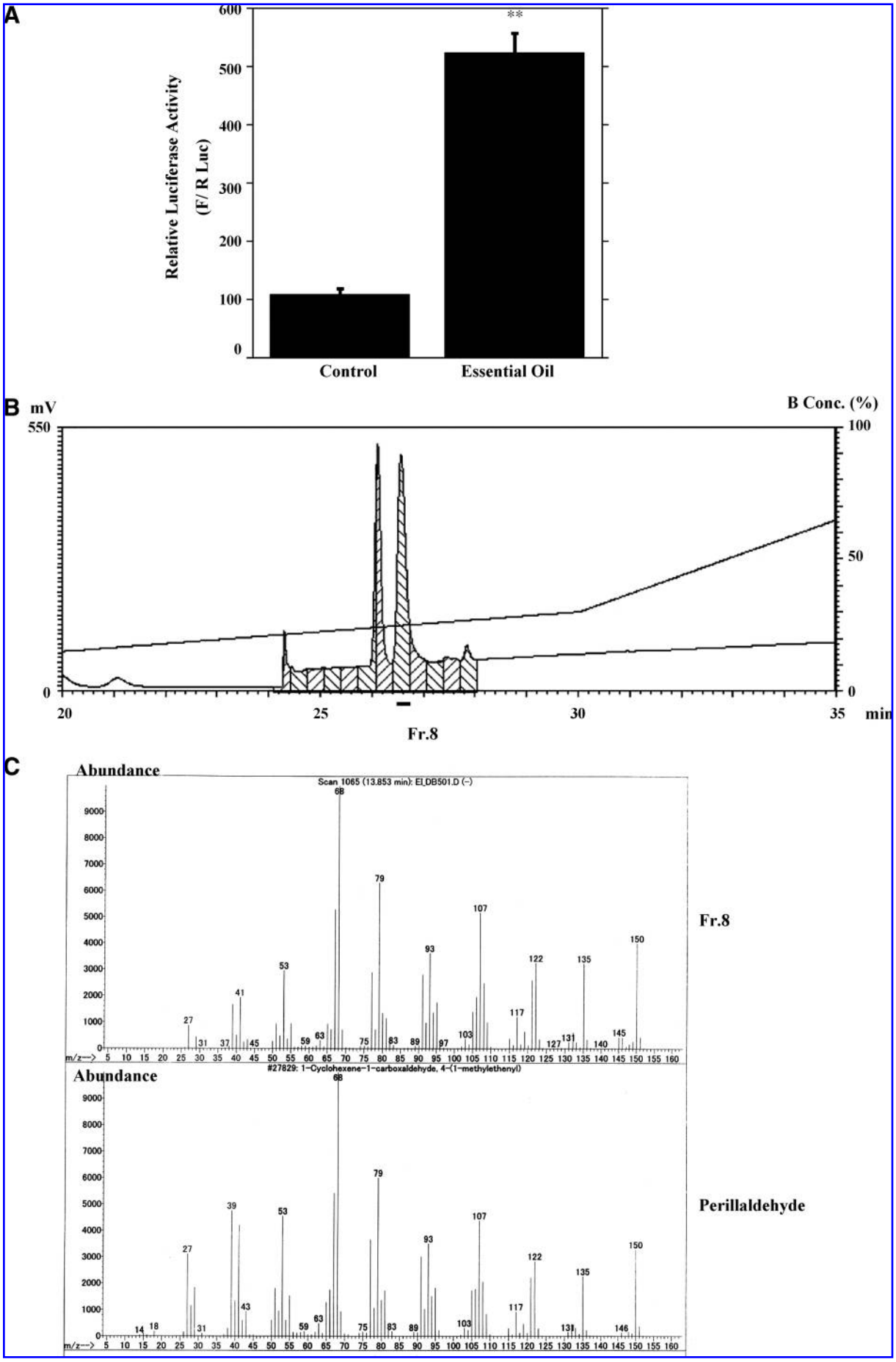
FIG. 1. Activation of the thioredoxin gene by extracts from Brassicaceae plants, *Psophocarpus tetragonolobus*, *Artemisia princeps*, and *Perilla frutescens*. K562 cells were transfected by using pTRX(-1148)-Luc or pGL3-basic and then treated with DMSO (*DMSO*), 10 μM sulforaphane (*SF*), ethanol (*EtOH*), or extracts from sprouts of red cabbage (**A**), rocket (**A**), DMSO (**B**, 1), 10 μM sulforaphane (**B**, 2), ethanol (**B**, 3), extracts from radish leaves (**B**, 4), daikon radish sprout (**B**, 5), Japanese horseradish leaves (**B**, 6) or roots (**B**, 7), ethanol (**B**, 8), or extracts from watercress (**B**, 9) *Perilla frutescens* (**C**, *Perilla Ext.*), *Psophocarpus tetragonolobus* (**C**, *winged bean*), soybean (**C**, *soybean*) or *Artemisia princeps* (**D**, *Mugwort* ext.), at a final concentration of 1% for 16 h, and luciferase activity was measured. To control for the efficiency of transfection, *Renilla* luciferase gene expression was monitored by using pRL-TK. The average level of *Firefly* luciferase activity was normalized to the level of *Renilla* luciferase activity (*F/R Luc*). Results are expressed as the mean ± standard deviation (SD) of triplicate samples from a representative experiment (**A**, **C**, and **D**) or duplicate samples from two independent experiments (**B**). Data were analyzed with Student's *t* test compared with controls and considered significant if a *p* value of <0.05 was observed. $^{*}p < 0.05$; $^{**}p < 0.01$.

thioredoxin gene through ARE. We further investigated the sensing mechanism of Keap1, comparing the actions of perillaldehyde and sulforaphane.

Materials and Methods

Cell lines and culture

l-Perillaldehyde (Nacalai), diluted in ethyl acetate (Nacalai), was used as perillaldehyde if not mentioned otherwise. (*S*)-(−)-perillaldehyde; (*R*)-(+)-perillaldehyde; (*S*)-(−)-perillyl alcohol; methyl perillate; (*S*)-(−)-perillic acid; *trans*-cinnamaldehyde; safranal; 2,4-octadienal; citral; octanal; nonanal; decanal; myrtenal; melonal; vanillin, *m*-, *o*-, and *p*-anisaldehyde; cumminaldehyde; *trans*-2, *cis*-6-nonadienal; *trans*-2-hexenal; Triton-X100; and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Sulforaphane was obtained from LKT Laboratories Inc. (*S*)-perillyl acetate was from Wako. Essential oil from *Perilla frutescens* was purchased from Tree of Life Co., Ltd (Tokyo). K562 cells were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal calf serum supplemented with antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin) at


A
Relative Luciferase Activity
(F/ R Luc)
600
500
400
300
200
100
0
**
Control
Essential Oil
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Perillaldehyde
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37°C in a humid atmosphere containing 5% CO_2. The 293 cells were maintained in Dulbecco's minimum essential medium (DMEM). RGM-1 cells, a rat gastric mucosal cell line, were kindly provided by Dr. Matsui of Tsukuba University. RGM-1 cells were cultured in DMEM/Ham's F-12 medium supplemented with 20% serum.

Preparation of extracts from vegetables

Extracts from Brassicaceae plants were prepared as described previously (41). Fresh vegetables were homogenized in two volumes of water and freeze dried. The freeze-dried material was dissolved in water or acetone. The mixture was filtered by a membrane filter and dried by evaporator. The dried material was dissolved in 1 ml of ethanol and used for analyses. The essential oil of *Perilla frutescens* was fractioned by preparative high-performance liquid chromatography (HPLC) by using an Inertsil SIL-100A (4.6×250 mm or 10×250 mm) (GL Sciences) column and a Shimazu HPLC system (Prominence, Shimazu, Kyoto, Japan). The flow solvent was a gradient of normal hexane/ethyl acetate at a flow rate of 1 or 5 ml/min. Detection was carried out at 260 nm. Gas chromatography/mass spectrometry analyses were performed by the TOSOH Analysis and Research Center (Manyo, Yamaguchi, Japan).

Plasmids

The pTrxCAT plasmids were constructed as described previously (33). The *Hind*III-*Bam*HI inserts from the pTrxCAT vectors were subcloned into pBluescriptII KS (+) (pTRXblue vectors). The pTRX-Luc vectors used in this study were prepared as described previously (14, 15). The pTRX (-1148)-Luc, pTRX (-874)-Luc, pTRX (-463)-Luc, pTRX (-352)-Luc, and pTRX (-76)-Luc vectors were constructed by ligating the *Kpn*I/*Bam*HI fragments of the pTRXblue vectors into the *Kpn*I/*Bgl*II sites of the pGL3 basic vector (Promega, Madison, WI). All constructs were controlled with direct nucleotide sequencing by using a BigDye terminator v3.1 cycle-sequencing kit (ABI). The pRL-TK vector was purchased from Promega. pcDNA3 was purchased from Invitrogen. The oligonucleotides used for construction of vectors were as follows: ARE-WT, 5′-cGGTCACCGTTACTCAGCACTTTG-3′ (forward), and 5′-ctagCAAAGTGCTGAGTAACGGTGACCggtac-3′ (reverse), ARE-M, 5′-cGGTCACCACCACCTTGCACTTTG-3′ (forward) and 5′-ctagCAAAGTGCAAGGTGGTGGTGACCggtac-3′ (reverse). pcDNA3-3X-ARE-Luc was constructed as follows.

The pGL3-Basic-TATA vector was constructed by ligating the *Eag*I-*Bam*HI fragment of pTrxCAT into the *Sma*I-*Bgl*II site of the pGL3 basic vector. Synthetic oligonucleotides containing the ARE sequence of the thioredoxin gene were inserted into the *Kpn*I-*Nhe*I site to produce pGL3-ARE-WT-Luc and pGL3-ARE-M-Luc. The *Kpn*I-*Hind*III site of pGL3-ARE-WT-Luc was inserted into the *Nru*I-*Hind*III site of pcDNA3 (Invitrogen) to produce pcDNA3-ARE-WT-Luc, into the *Pvu*I-*Nhe*I site of which synthetic oligonucleotides containing three tandem repeats of the ARE sequence of the thioredoxin gene were inserted. Then the *Bgl*II-*Xba*I fragment of pGL3-Basic was inserted into the *Bam*HI-*Xba*I site of the vector to produce pcDNA3-3X-ARE-Luc. The pCMV-Tag2 vector was purchased from Stratagene. Keap1 cDNA (pF1KADA0132) was purchased from Kazusa DNA Research Institute, amplified by polymerase chain reaction (PCR) by using KOD plus and cloned into pCR-BluntII-TOPO.

The oligonucleotides used for the PCR were as follows: GGATCCAGGAGATAGAACCATGCAGCCA (forward) and CTCGAGAACAGGTACAGTTCTGCTGGTC (reverse). The *Bam*HI/ *Xho*I fragment of the TOPO vector was ligated into the *Bam*HI/*Xho*I site of the pCMV-Tag2 vector to produce pCMV-Tag2-Keap1. R272A oligonucleotides were used to produce pCMV-Tag2A-Keap1-R272A. The vector and K287A oligonucleotides were used to produce pCMV-Tag2A-Keap1-R272A/K287A. C273A oligonucleotides were used to produce pCMV-Tag2A-Keap1-C273A. C288A oligonucleotides and the pCMV-Tag2A-Keap1-C273A vector were used to produce pCMV-Tag2A-Keap1-C273A/C288A. The oligonucleotides used for the PCR were as follows: Keap1 R272A, CGGGCC GTGGCCTGCCACTCGTTGAC (forward), GTCAACGAGT GGCAGGCCACGGCCCG (reverse); Keap1 K287A: GATGC AGCTGCAGGCCTGCGAGATCCTGCAGTC (forward) and GACTGCAGGATCTCGCAGGCCTGCAGCTGCATC (reverse); Keap1 C273A, CCGTGCGCGCCCACTCGTTGAC GCC (forward) and GGCGTCAACGAGTGGGCGCGCACGG (reverse); and Keap1 C288A, GCAGCTGCAGAAGGCCGAG ATCCTGCAGTC (forward) and GACTGCAGGATCTCGGC CTTCTGCAGCTGC (reverse).

Transfection and luciferase assay

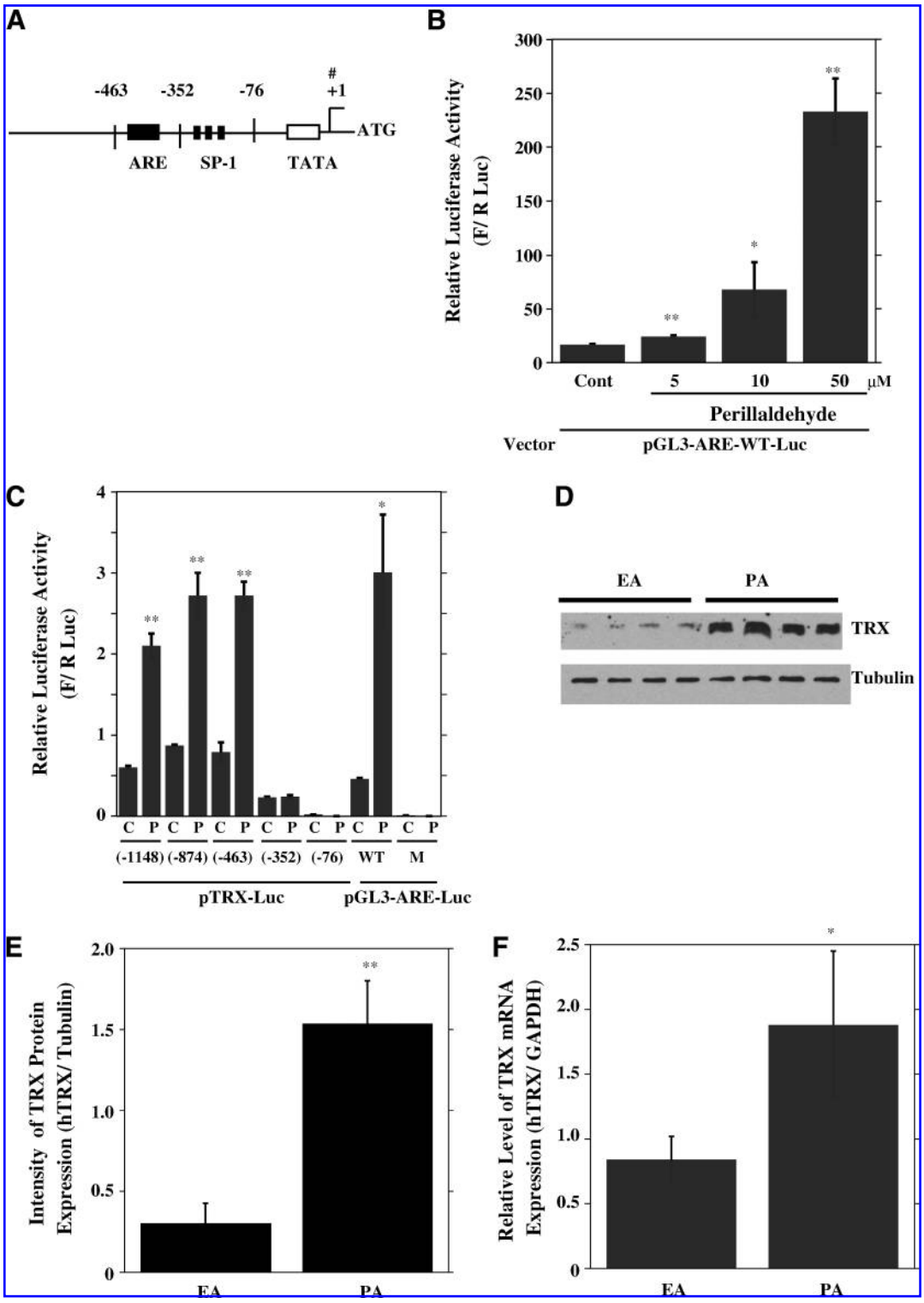
K562 cells were transfected with luciferase reporter expression vectors by using DMRIE-C (GIBCO) according to the manufacturer's instructions. After 4 h of incubation, sample or control was added. To control the efficiency of transfection, *Renilla* luciferase gene expression was monitored by using pRL-TK (Promega). Luciferase gene expression was analyzed 24 h later by using an assay kit (Promega). Assays were performed in duplicate. In total, 293 cells were transfected with Trans-It-LT1 (Mirus). For the treatment with

FIG. 2. The identification of thioredoxin-inducing substances in *Perilla frutescens*. (**A**) Activation of the thioredoxin gene by essential oil of *Perilla frutescens*. K562 cells were transfected with pcDNA3-3X-ARE-Luc and then treated with ethanol (*Control*), or essential oil of *Perilla frutescens* (diluted in ethanol 1:5,000) at a final concentration of 1%. After 16 h, luciferase activity was measured. The average level of *Firefly* luciferase activity normalized to the level of *Renilla* luciferase activity (*F/R Luc*) with SD is shown. Data were analyzed with Student's *t* test compared with controls and considered significant if a *p* value of <0.05 was observed. $^{**}p < 0.01$. (**B**) Purification of constituents containing thioredoxin-inducing activity. Essential oil of *Perilla frutescens* was analyzed with HPLC by using an Inertsil-SIL 100A column (10×250 mm, GL Science) in a gradient of hexane/ethylacetate at a flow rate of 5 ml/min. The fraction containing thioredoxin-inducing activity was reanalyzed with HPLC by using the Inertsil-SIL 100A column in a hexane/ethyl acetate solution. (**C**) Mass spectrometric analyses of the constituent of the HPLC fraction containing the most thioredoxin-inducing activity. The fraction containing thioredoxin-inducing activity (Fr. 8 in Fig. 2B) was analyzed with gas chromatography by using Agilent GI-800A. *Upper panel*: Mass spectrometric pattern of Fr. 8 in Fig. 2B. *Lower panel*: Mass spectrometric pattern of perillaldehyde.

perillaldehyde, the same concentration of ethyl acetate was used as a control.

Semiquantitative reverse transcription (RT)-PCR

For the thioredoxin mRNA expression analyses, total RNA was isolated from the cells by using Trizol reagent (Invitrogen) or an RNeasy kit (Qiagen). cDNA was synthesized by using a SuperScript First-Strand Synthesis System (Invitrogen) with oligo $dT_{12–18}$. The primers used for the amplification were as follows: human thioredoxin, 5′-ATGGTGAAGCAG ATCGAG-3′ (forward) and 5′-TTAGACTAATTCATTAAT GGT-3′ (reverse); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ATGGGGAAGGTGAAGGTCGGA GTC-3′ (forward) and 5′-CCATGCCAGTGAGCTTCCCG TTC-3′ (reverse). PCR was performed under the following



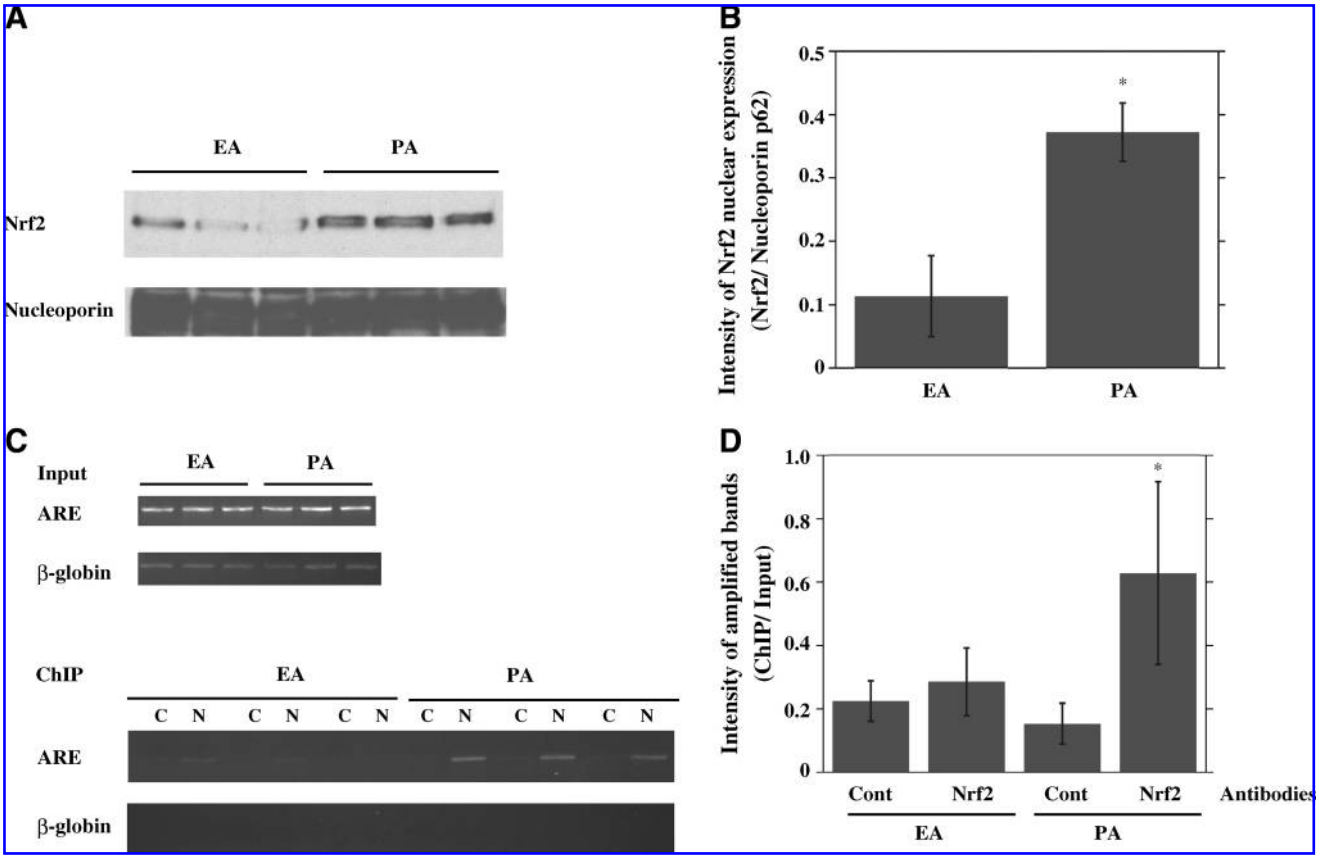


FIG. 4. Increased binding of Nrf2 to the ARE of the thioredoxin gene after perillaldehyde treatment. (**A**) Nuclear expression of Nrf2. Nuclear extracts from K562 cells treated with 50 μM perillaldehyde for 6 h were analyzed with Western blotting by using anti-Nrf2 (H-300) or anti-nucleoporin p62 (BD biosciences) antibodies. *EA,* ethylacetate (0.1%); *PA,* perillaldehyde. (**B**) Densitometric analyses of the Western blotting results. The intensities of the bands detected with anti-Nrf2 antibodies shown in (**A**) were normalized to those detected with anti-nucleoporin p62, as analyzed with Image-J software and shown as an average of samples with SD. (**C**) Chromatin immunoprecipitation assay. K562 cells were treated with 50 μM perillaldehyde for 3 h and subjected to chromatin immunoprecipitation assays by using probes targeting the ARE of the thioredoxin gene (*upper panel*) or the coding region of the β-globin gene (*lower panel*). *C,* control IgG; *N,* anti-Nrf2 antibody. The PCR-amplified input of each sample also is shown. (**D**) Densitometric analyses of the PCR results. The intensities of PCR bands shown in (**C**) were normalized to those of the inputs, as analyzed with Image-J software, and shown as an average of samples with SD. Data were analyzed with Student's *t* test compared with controls and considered significant if a *p* value of <0.05 was observed. $^*p < 0.05$.

FIG. 3. Activation of the thioredoxin gene by perillaldehyde. (**A**) An illustration of the thioredoxin promoter. The transcriptional start point proximal to the first codon (#) was attributed as +1. The positions of the Sp1 binding sites, antioxidant responsive element (ARE) and the TATA box, as well as the region contained in the deletion mutants of the thioredoxin promoter luciferase constructs, are shown. (**B**) Dose-dependent activation of the thioredoxin gene by perillaldehyde. K562 cells were transfected by using pGL3-ARE-WT-Luc and then treated with ethyl acetate (*Cont*) or 5, 10, or 50 μM perillaldehyde for 16 h. Relative luciferase activity normalized to *Renilla* luciferase activity with SD is shown. (**C**) Activation of the thioredoxin gene by perillaldehyde *via* the region containing the ARE. K562 cells were transfected with pTRX (−1148)-Luc, pTRX (−874)-Luc, pTRX (−463)-Luc, pTRX (−352)-Luc, pTRX (−76)-Luc, pGL3-ARE-WT-Luc, or pGL3-ARE-M-Luc, and then treated with ethyl acetate (*C*) or 50 μM perillaldehyde (*P*) for 16 h. Relative luciferase activity normalized to *Renilla* luciferase activity with SD is shown. (**D**) Induction of thioredoxin expression by perillaldehyde. The expression of thioredoxin protein in ethyl acetate (*EA*) or perillaldehyde (*PA*) (50 μM for 48 h)-treated K562 cells is shown. Whole-cell lysates (1 μg) from quadruplicated samples were loaded on a 15% SDS-PAGE gel and detected by using anti-thioredoxin (*TRX*) or anti-tubulin monoclonal antibodies. (*S*)-(−)-perillaldehyde (Aldrich) was used in the experiment. (**E**) Densitometric analyses of the expression of thioredoxin. The relative levels of the expression of thioredoxin (*TRX*) shown in (**C**) were normalized to those of tubulin, as analyzed with Image-J software, and shown as an average of samples with SD. (**F**) Induction of thioredoxin mRNA expression by perillaldehyde analyzed with real-time RT-PCR. Real-time RT-PCR analyses of K562 cells treated with 0.1% ethyl acetate (*EA*) or 50 μM perillaldehyde (*PA*) for 16 h were performed. The relative level of expression of thioredoxin normalized to the amount of GAPDH is shown as an average of triplicate samples with SD. Data were analyzed with Student's *t* test compared with controls and considered significant if a *p* value of <0.05 was observed. $^*p < 0.05$; $^{**}p < 0.01$.

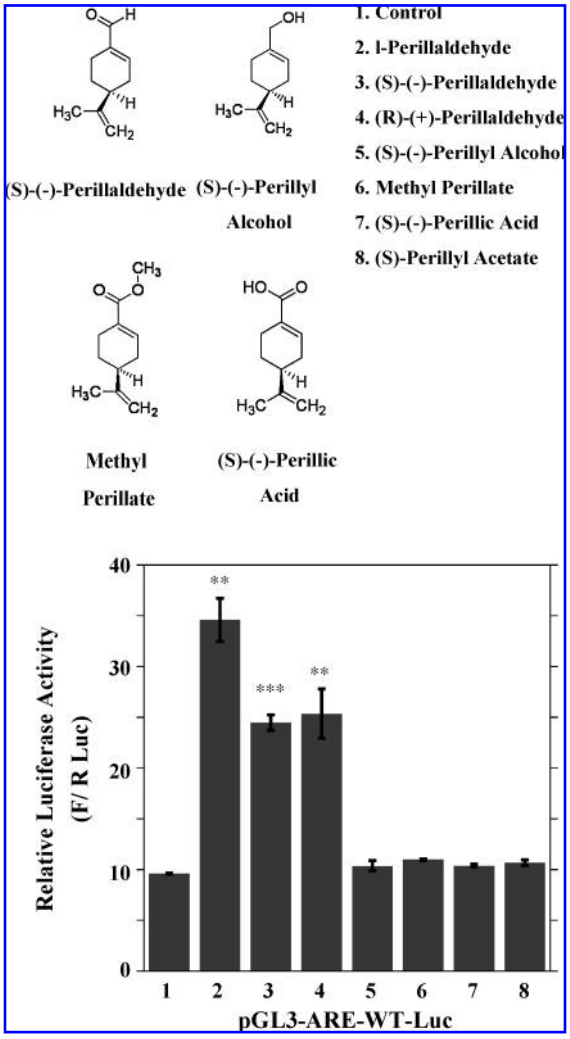


FIG. 5. The aldehyde moiety of perillaldehyde is responsible for ARE activation. K562 cells were transfected with pGL3-ARE-WT-Luc and then treated with ethyl acetate (*Control*), 10 μM perillaldehyde, or the reagents indicated, for 16 h. The relative luciferase activity of triplicate samples normalized to *Renilla* luciferase activity with SD is shown. Data were analyzed with Student's t test compared with controls and considered significant if a p value of <0.05 was observed. $^{**}p < 0.01$; $^{***}p < 0.001$.

conditions: 20 cycles for human thioredoxin (94°C for 30 s, 50°C for 30 s, and 72°C for 1 min), and 22 cycles for human GAPDH (94°C for 30 s, 61°C for 1 min, and 72°C for 90 s). The PCR products were visualized with electrophoresis in agarose gels. Real-time PCR was performed by using Takara SYBR Premix Ex Taq.

Western blot analyses

Cells were collected and washed twice with ice-cold phosphate-buffered saline (PBS), and then lysed with a solubilizing solution [20 m*M* Tris-HCI (pH 7.5), 150 m*M* NaCl, 1%Triton X-100, 1 m*M* EDTA, 1 m*M* EGTA, 2.5 m*M* sodium pyrophosphate, 1 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4. and protease inhibitor cocktail (Roche)] on ice for 10 min and sonicated. The extracts were cleared by centrifugation. Cell lysates were kept at 95°C for 5 min and then separated by 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA). The membrane was treated with 10% (wt/vol) skim milk in T-PBS (PBS, containing 0.05% Tween20) overnight, and incubated with anti-human thioredoxin monoclonal antibody (Redox Bioscience, Inc.) for 1 h, followed by peroxidase-conjugated anti-mouse IgG (dilution, 1:2,000) (Amershan Pharmacia Biotech) for 1 h. The epitope was visualized with an ECL Western blot detection kit (Amershan Pharmacia Biotech). Monoclonal anti-tubulin antibodies (Sigma, Saint Louis, MO) were used to control for the amounts of protein loaded. Nuclear extracts were prepared as described previously (15).

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed by using a One Day ChIP kit (Diagenode, Belgium). K562 cells were crosslinked with 1% formaldehyde in PBS for 5 min at room temperature. The cells were washed twice with ice-cold PBS and lysed with SDS lysis buffer. Chromatin was sheared by sonication (6 times for 30 s each time with 60-s cooling in between) by using a Bioruptor (Cosmo Bio Co. Ltd., Tokyo), centrifuged, and incubated overnight with anti-Nrf2 antibody (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG (Santa Cruz Biotechnology) at 4°C. The immunoprecipitated DNA was analyzed with PCR by using Taq DNA Polymerase Mix (Sigma) with the following promoter-specific primers: thioredoxin, 5′-GACGTACACACCGAGATA-3′ (forward) and 5′-ATCAGCACTGCGCGTGA-3′ (reverse): and human β-globin, 5′-GGCAAGGTGAACGTGGATGAAGTTGGTG-3′ (forward) and 5′-GGAGTGGACAGATCCCCAAAGGACT CAAAG-3′ (reverse). The primers for human β-globin were used to amplify a 237-bp segment of the coding region. The PCR conditions were as follows: 95°C for 3 min; 36 cycles at 95°C for 30 s; 53°C for 1 min; 72°C for 2 min; and a final elongation at 72°C for 8 min to amplify a 248-bp stretch of the human thioredoxin promoter containing the ARE region. The PCR conditions to amplify the internal control were as follows: 94°C for 3 min; 27 cycles at 94°C for 30 s; 66°C for 1 min; 72°C for 2 min; and a final elongation at 72°C for 8 min.

Results

Enhancement of thioredoxin expression by extracts from Perilla frutescens

We assessed whether activation of the thioredoxin gene is changed by treatment of K562 cells with extracts from 76 edible Japanese vegetables (Table 1). Among the extracts tested, those from Brassicaceae plants, such as broccoli sprout (data not shown), red cabbage sprout, rocket, radish, daikon radish sprout and Japanese horseradish leaves and root, and watercress showed thioredoxin-inducing activity (Fig. 1A and B), as reported in or anticipated from previous reports (4, 25). We also showed that the extracts from *Perilla frutescens* (perilla, Ao-siso), *Artemisia princeps* (mugwort), and *Psophocarpus tetragonolobus* (winged bean) activated expression of the thioredoxin gene (Fig. 1), but not that of a control reporter (data not shown), and induced the expression of thioredoxin mRNA and protein (data not shown). Although isothio-

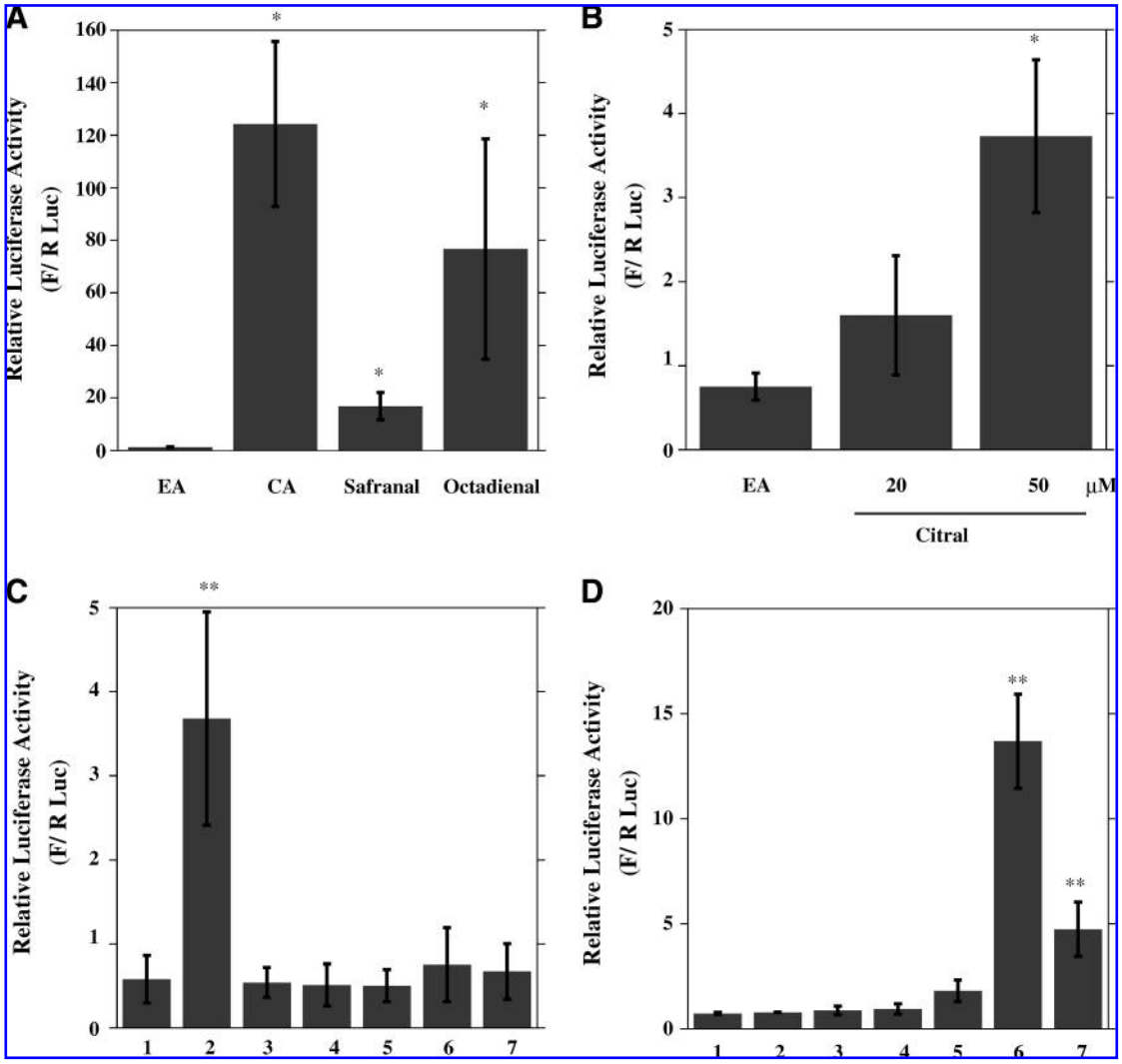


FIG. 6. Unsaturated aldehydes are inducers of thioredoxin. K562 cells were transfected by using pcDNA3-3X-ARE-Luc and then treated with ethyl acetate (*EA*) or 20 μM cinnnamaldehyde (*CA*, **A**), 20 μM safranal (**A**), or 20 μM 2, 4-octadienal (**A**), and the indicated doses of citral (**B**), ethylacetate (**C**, 1), and 50 μM perillaldehyde (**C**, 2), 50 μM octanal (**C**, 3), 50 μM nonanal (**C**, 4), 50 μM decanal (**C**, 5), 50 μM myrtenal (**C**, 6), or 50 μM melonal (**C**, 7), ethyl acetate (**D**, 1), 20 μM vanillin (**D**, 2), 20 μM *m*-anisaldehyde (**D**, 3), 20 μM *o*-anisaldehyde (**D**, 4), or 20 μM *p*-anisaldehyde (**D**, 5), 20 μM *trans*-2-*cis*-6-nonadienal (**D**, 6), or 20 μM *trans*-2-hexenal (**D**, 7), and then luciferase assays were performed. Relative luciferase activity normalized to *Renilla* luciferase activity is shown. Results are expressed as the mean $\pm$ SD of triplicate samples from a representative experiment (**A** and **B**) or duplicate samples from two independent experiments (**C** and **D**). Data were analyzed with Student's *t* test compared with controls and considered significant if a *p* value of <0.05 was observed. $^{*}p < 0.05$; $^{**}p < 0.01$.

cyanates in the extracts from Brassicaceae plants seem to be responsible for this thioredoxin-inducing activity, we were unable to detect isothiocyanates in the extracts from *Perilla frutescens* and *Artemisia princeps* (data not shown). The extracts from *Perilla frutescens* and *Artemisia princeps* activated the thioredoxin reporter construct containing wild-type ARE, but not the construct containing mutant ARE (data not shown), suggesting that the thioredoxin inducers activate expression of the thioredoxin gene through the ARE.

We then performed activity-guided purification of the extract of *Perilla frutescens* by using a luciferase reporter vector (pcDNA3-3X-ARE-Luc) containing three tandem repeats of the ARE of the thioredoxin gene. Fresh leaves of *Perilla frutescens* (200 g) were extracted with 1 L of ethyl acetate overnight at room temperature. After evaporation, the extract was redissolved in an adequate volume of ethyl acetate, which was applied to a carbon graphite column. The pass-through portion was fractionated by flash-column chromatography on silica gel with a step gradient of *n*-hexane/ethylacetate. The 30% ethyl acetate fraction was further fractioned with preparative HPLC to show that the extract contains activity to activate ARE (data not shown). However, we obtained little activity to activate ARE with further purification.

Because the possibility existed that volatile substances are responsible for the activity, we then tested the essential oil from *Perilla frutescens.* As expected, it too induced the activation of a vector containing ARE (Fig. 2A), but not the PGL3 basic vector (data not shown).

We next performed activity-guiding purification of the essential oil. An HPLC fraction containing the most

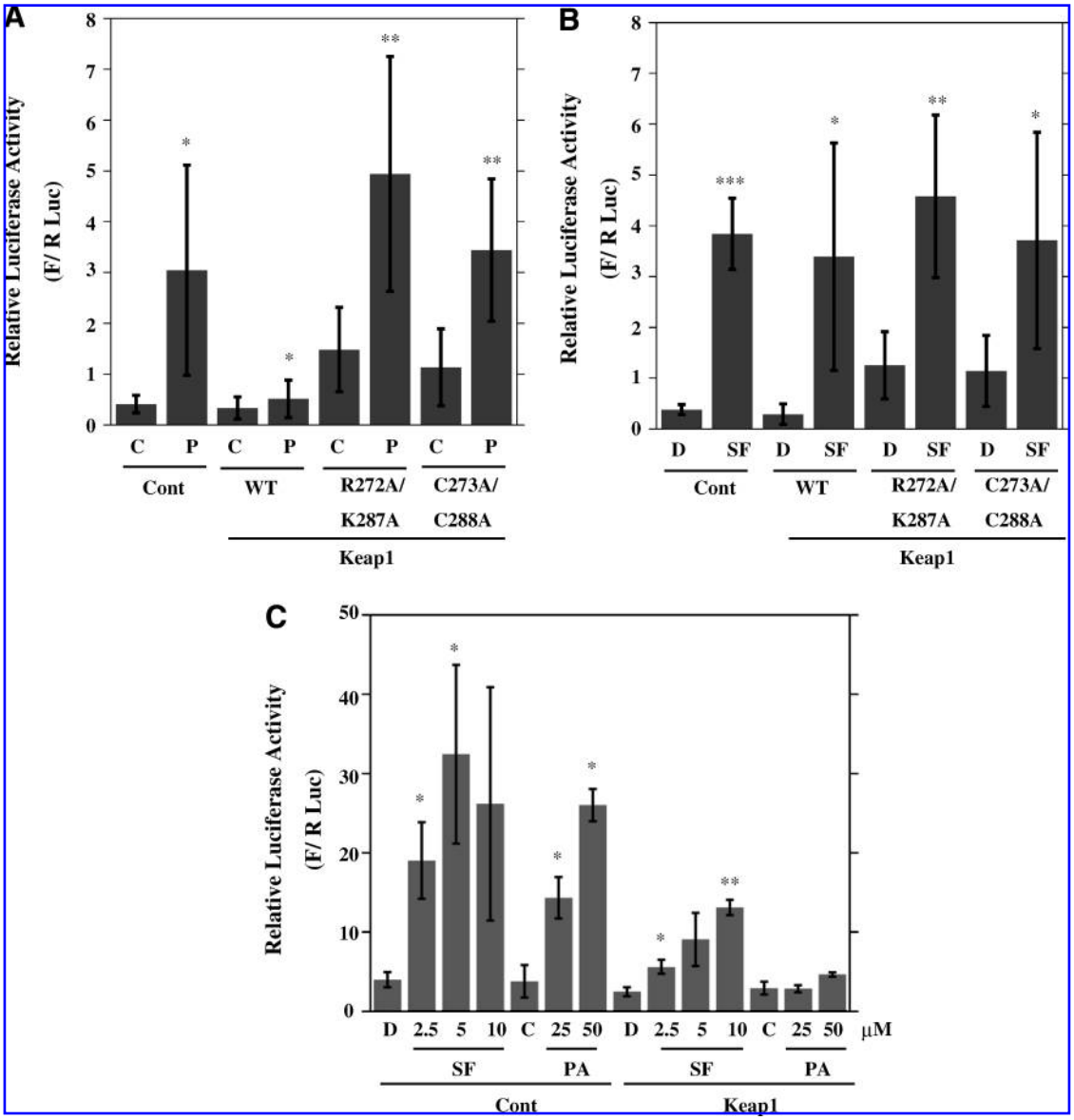


FIG. 7. Suppression of perillaldehyde-induced activation through the ARE by Keap1. The 293 cells were transfected with the indicated vectors, together with pGL3-ARE-WT-Luc, and then treated with ethyl acetate (*C*), 50 μM perillaldehyde (*P*), DMSO (*D*), 10 μM sulforaphane (*SF*), or the indicated concentrations of sulforaphane or perillaldehyde for 16 h. Relative luciferase activity normalized to *Renilla* luciferase activity is shown. The results are shown as an average with SD of duplicate samples from two independent experiments (**A** and **B**), or triplicate samples (**C**). Data were analyzed with Student's *t* test, compared with controls, and considered significant if a *p* value of <0.05 was observed. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

thioredoxin-inducing activity (Fig. 2B) was subjected to gas chromatography/mass spectrometry analyses. The molecular mass of the constituent in the fraction was revealed to be 150 Da, and the mass spectrometric pattern was identical to that for perillaldehyde (Fig. 2C). These results strongly indicate that the constituent responsible for the thioredoxin-inducing activity in *Perilla frutescens* is perillaldehyde.

Perillaldehyde-induced thioredoxin expression

We then tested whether perillaldehyde induces thioredoxin expression. At concentrations of 10–50 μM, perillaldehyde activated pTRX-ARE-WT-Luc luciferase reporter genes in a dose-dependent manner (Fig. 3B), but not the pGL3-basic vector (data not shown). Perillaldehyde treatment activated luciferase reporter genes such as pTRX (-1148)-Luc, pTRX (-874)-Luc, pTRX (-463)-Luc, and pTRX-ARE-WT-Luc but not pTRX (-352)-Luc, pTRX (-76)-Luc, or pTRX-ARE-M-Luc (Fig. 3C). Perillaldehyde induced protein expression of thioredoxin in K562 (Fig. 3D and E) and HepG2 cells (data not shown) and mRNA expression in K562 cells, as analyzed with real-time RT-PCR assay (Fig. 3F). The expression of glutamate cysteine ligase catalytic subunit (GCLC) and NAD(P)H dehydrogenase, quinone 1 protein was not significantly changed by perillaldehyde treatment in K562 cells.

We further analyzed the effect of perillaldehyde on the expression of thioredoxin in mice. Oral administration of perillaldehyde to mice induced slight expression of thior-

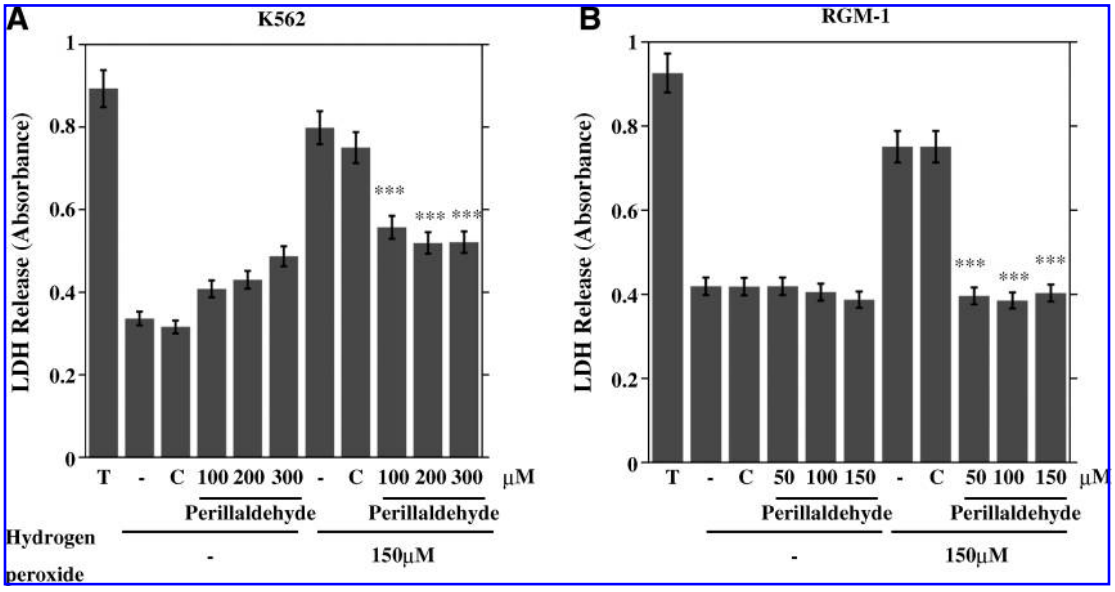


FIG. 8. Attenuation of hydrogen peroxide–induced cellular damage by perillaldehyde. K562 (**A**) or RGM-1 (**B**) cells were not treated (−) or pretreated with 0.05% ethyl acetate (*C*) or the indicated concentration of perillaldehyde, and then treated with the indicated concentration of hydrogen peroxide. Release of LDH was measured as described in Materials and Methods. Optical density determined with 2% Triton-X100 (*T*) is attributed to 100% cell killing. Results are expressed as the mean ± SD of six samples from a representative experiment. Data were analyzed with Student's *t* test compared with controls and considered significant if a p value of <0.05 was observed. ***$p < 0.001$.

edoxin mRNA and significant expression of GSTα, although the dose was as high as 18 mg/body/day (data not shown). We also showed that perillaldehyde or sulforaphane augmented the nuclear expression of Nrf2 protein (Fig. 4A and B). We examined whether perillaldehyde induces the binding of Nrf2 to the ARE by using a chromatin immunoprecipitation (ChIP) assay. Perillaldehyde significantly induced a PCR signal from precipitates obtained by using anti-Nrf2 antibodies, when the primer sets were used to amplify the ARE region of the thioredoxin gene. By contrast, perillaldehyde did not change the signal obtained by using the control primer sets or control antibodies (Fig. 4C and D). These results demonstrated that perillaldehyde induces the expression of the thioredoxin gene *via* the ARE region of its promoter.

The aldehyde moiety of perillaldehyde is responsible for thioredoxin-inducing activity

We next analyzed which moiety of the structure of perillaldehyde is responsible for the thioredoxin-inducing activity. We first tested compounds related to perillaldehyde. Perillyl alcohol, methyl perillate, perillic acid, perillyl acetate (Fig. 5), and limonene (data not shown) had no significant thioredoxin-inducing activities, suggesting that the aldehyde moiety of perillaldehyde is responsible for the thioredoxin-inducing activity. Both the (*R*)- and (*S*)- forms of perillaldehyde induced ARE activation. Aldehyde compounds from plants, such as *trans*-cinnamaldehyde, safranal, 2,4-octadienal (Fig. 6A), citral (Fig. 6B), *trans*-2-*cis*-6-nonadienal, and *trans*-2-hexenal (Fig. 6D), showed activation through ARE. Essential oils from *Cymbopogon citrarus* also possessed the ability to activate the ARE (data not shown). However, aldehydes such as octanal, nonanal, decanal (Fig. 6C), undecanal (data not shown), myrtenal, melonal (Fig. 6C), vanillin, anisaldehyde (Fig. 6D), acetaldehyde, piperonal, cumminaldehyde, benzaldehyde, and glyceraldehyde (data not shown) did not show any ability to activate the ARE. Acrolein and 4-hydroxy-2-nonenal, but not propionaldehyde, induced ARE activation (data not shown). These results suggest that aldehydes containing α, β-unsaturated aldehydes have the ability to activate AREs.

Perillaldehyde-induced activation via the ARE is suppressed by Keap1

Electrophilic substances are considered to interact with Keap1 to stabilize Nrf2 protein and subsequently to activate AREs (16). Cysteine residues 273 and 288 are shown to be essential for Keap1 to repress Nrf2 activity *in vivo* (38). We tested the effect of overexpression of Keap1 on perillaldehyde-induced activation of the ARE by using 293 cells with high transfection efficiency. Overexpression of wild-type Keap1 almost completely suppressed perillaldehyde-induced activation through ARE, whereas the sulforaphane-induced activation seemed to be partially suppressed (Fig. 7A–C). By contrast, overexpression of Keap1 mutants, such as Keap1 R272A/ K287A and Keap1 C273A/C288A, did not suppress the perillaldehyde- and sulforaphane-induced activation (Fig. 7A and B). These results suggest that perillaldehyde activates the Keap1/Nrf2 system, possibly through its interaction with amino acids around critical cysteine residues.

Induction of thioredoxin by perillaldehyde pretreatment rescues oxidative stress–induced cellular damage

We further examined whether pretreatment of K562 cells and RGM-1 cells with perillaldehyde could reduce oxidative stress–induced damage by analyzing the release of LDH from damaged cells (Fig. 8A and B). The pretreatment

reduced hydrogen peroxide–induced LDH release, whereas perillaldehyde treatment alone scarcely augmented LDH release, suggesting that the pretreatment with perillaldehyde is protective against oxidative stress–induced cellular damage.

Discussion

We detected thioredoxin-inducing activity in extracts from several cruciferous plants, *Perilla frutescens*, *Artemisia princes*, and *Psophocarpus tetragonolobus*. Because the extracts from *Perilla frutescens* and *Artemisia princeps* do not contain isothiocyanates (data not shown), they seem to contain novel thioredoxin-inducing and ARE-activating substances. The active constituent from *Artemisia princeps* was purified and seems to be unique (Takatera *et al*., data not shown). In the present study, we demonstrated that perillaldehyde, a fragrant substance in *Perilla frutescens*, has strong thioredoxin-inducing activity. The expression of GCLC and NAD(P)H dehydrogenase, quinone 1 protein, was not significantly changed by perillaldehyde treatment in K562 cells. The differences of the mode of the regulation of various genes under the control of AREs may depend on cell types and should be further investigated. Both (*S*)-(−)-perillaldehyde and (*R*)-(+)-perillaldehyde have the ability to activate AREs, indicating that chirality does not change this effect (Fig. 5). Among the aldehydes tested, only those containing an α, β-unsaturated aldehyde moiety showed a significant ability to activate the ARE (Fig. 6). These compounds seem to have electrophilic potential. A well-known example of such a reaction is seen in harmful aldehydes, such as acrolein and 4-hydroxy-2-nonenal (36, 37), which are often produced during lipid peroxidation. Acrolein and 4-hydroxy-2-nonenal were reported to induce the expression of GST-P (6) and activation of Nrf2 (17, 35). Acrolein is also reported to induce the expression of thioredoxin reductase in human umbilical vein endothelial cells (28). Thus, it is possible that aldehyde compounds in plants mimic the gaseous aldehydes, which mediate signals generated in cellular metabolic cycles, such as lipid peroxidation, leading to activation of Nrf2/ARE and the upregulation of redox enzymes. Although the direct modification of cysteine residues by electrophiles is proposed as a mechanism for the activation of the Nrf2/Keap1 axis, little is known of how oxidative stress is properly transmitted to the Nrf2/Keap1 sensor in the cytosol containing rich amounts of glutathione and thioredoxin family proteins. Aldehydes themselves or some cytosolic constituents modified by aldehydes may interact with Keap1. It was shown recently that 8-nitro-cGMP, a cGMP derivative modified by nitric oxide, causes the modification of Keap1 (30). As Keap1 is proposed to be a zinc metalloprotein (2), aldehydes may interact with the zinc-containing sensor of Keap1. A more widely accepted view is that the C273 and C288 of Keap1 are highly active cysteine residues (38). Here, we showed that overexpression of Keap1 also suppresses perillaldehyde-induced activation of AREs, whereas the sulforaphane-induced activation seemed to be partially suppressed. Perillaldehyde may activate the Keap1/Nrf2 system differently from sulforaphane. It was reported very recently that fish have two types of Keap1 (19). Although sulforaphane is reported to interact with Keap1 *in vitro* (3), it might be that the Nrf2/ARE system is differentially regulated by Broad complex/Tramtrack/Bric-a-brac (BTB)-Kelch family members in response to a wide variety of electrophiles. Overexpression of Keap1 mutants, such as Keap1 R272A/K287A and Keap1 C273A/C288A, did not suppress the activation (Fig. 7). These results suggest that the cysteine residues of the intervening region of Keap1 are required for perillaldehyde-induced activation, consistent with the previous view. In addition, lysine and arginine residues juxtaposed to the critical cysteine residues of Keap1 also seem to be important for the activation. K287 may function as an important residue for local electrostatic environment, or K287 might be directly modified. If one considers other possibilities, lysine residues within the Nrf2-ECH homology (Neh) 2 domain of Nrf2 are targets for ubiquitin transfer (40) and could be targets for aldehydes. Keap1 is reported to be modified by polyubiquitination at Lys-48 and Lys-298 (10), with which aldehydes could interact. Aldehydes might directly target the three arginine residues (Arg380, Arg415, Arg483) of Keap1 required for binding to Nrf2 (20) to hinder the Nrf2-Keap1 interaction. Such possibilities could also be tested. We are now preparing to analyze the modification of Keap1 and Nrf2 by perillaldehyde *in vivo*, by using mass fingerprinting analysis.

Aldehyde compounds, such as *trans*-cinnamaldehyde, safranal, 2,4-octadienal (Fig. 6A), citral (Fig. 6B), *trans*-2-*cis*-6-nonadienal, and *trans*-2-hexenal (Fig. 6D), all have a molecular mass of around 150 Da and are ligands for odorant receptors. The thioredoxin-inducing activities of these compounds were not suppressed by treatment with 2′,5′-dideoxyadenosine, suramin sodium, LY83,583, or 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA/AM) (data not shown), suggesting that the activities are independent of signals downstream of G-coupled protein receptors. Nevertheless, it is interesting to speculate that these fragrant aldehydes evoke not only odor perception, but also Nrf2/ARE signaling in mucosal cells. Perillaldehyde and other aldehyde compounds may cause oxidative stress, because they are highly active with thiol groups of proteins and behave as prooxidants. However, we showed here that pretreatment of cells with perillaldehyde suppresses the hydrogen peroxide–induced cellular damage. These results suggest that fragrant aldehydes, such as perillaldehyde, are less harmful than toxic aldehydes, such as acrolein, and may protect cells as long as they are used in small and suitable amounts, by inducing the expression of thioredoxin and other genes under the control of ARE.

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Abbreviations

ARE, antioxidant responsive element; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra-

kis (acetoxymethyl ester); BTB, Broad complex/Tramtrack/Bric-a-brac; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's minimum essential medium, DMSO, dimethyl sulfoxide; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GCLC, glutamate cysteine ligase catalytic subunit; GST, glutathione *S*-transferase; HPLC, high-performance liquid chromatography; KEAP1, Kelch-like ECH-associated protein 1; LDH, lactate dehydrogenase; Neh2, Nrf2-ECH homology 2; Nrf2, nuclear factor erythroid-2 related factor 2; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RT, reverse transcription; SD, standard deviation; SDS, sodium dodecylsulfate.

Disclosure Statement

No competing financial interests exist.

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