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Peroxynitrite Scavenging Activity of Sinapic Acid (3,5-Dimethoxy-4-hydroxycinnamic Acid) Isolated from *Brassica juncea*

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Peroxynitrite ($ONOO^-$), formed from a reaction of superoxide and nitric oxide, is one of the most potent cytotoxic species that are known to oxidize cellular constituents including essential proteins, lipids, and DNA. In this study, the ability of sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid), isolated from *Brassica juncea*, to scavenge $ONOO^-$ was investigated. The data obtained show that sinapic acid can efficiently scavenge native $ONOO^-$ as well as $ONOO^-$ derived from the peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1). Spectrophotometric analyses revealed that sinapic acid suppressed the formation of $ONOO^-$-mediated tyrosine nitration through an electron donation mechanism. In further studies, sinapic acid also showed a significant ability of inhibiting nitration of bovine serum albumin and low-density lipoprotein (LDL) in a dose-dependent manner. Sinapic acid decreased the LDL peroxidation induced by SIN-1-derived $ONOO^-$. The present study suggests that sinapic acid has an efficient $ONOO^-$ scavenging ability, which may well be a potent $ONOO^-$ oxidant scavenger for the protection of the cellular defense activity against the $ONOO^-$-involved diseases.

KEYWORDS: ***Brassica juncea*; sinapic acid; 3,5-dimethoxy-4-hydroxycinnamic acid; nitrotyrosine; $ONOO^-$ scavenging activity; lipid peroxidation**

INTRODUCTION

Peroxynitrite ($ONOO^-$), a potent oxidizing nitrating species, can be produced in vivo by the rapid interaction (second-order rate constant for reaction is 6.7×10^9 M^{-1} s^{-1}) (*1*) between the superoxide anion radical ($O_2^{\bullet -}$) and nitrogen monoxide ($^\bullet NO$). Since these two reactive species are known to be generated during inflammatory reactions with concomitant activation of NADPH oxidase and inducible NO synthases, the involvement of $ONOO^-$ in the proinflammatory process might be of great importance.

Part of $ONOO^-$'s physiological roles is the essential defensive process against invading microorganisms in vivo. In terms of the oxidative power, $ONOO^-$ is much more reactive, i.e., more toxic than $O_2^{\bullet -}$ or H_2O_2, as demonstrated in the cultured cells (*2*). Thus, an overproduction of or uncontrolled $ONOO^-$ could lead to cell death and tissue destruction. The generation of $ONOO^-$ has been suggested as a causative factor responsible for the mucosal injury in several gastrointestinal disorders (*3*).

At the cellular levels, it has been shown that $ONOO^-$ causes oxidation and nitration of proteins and guanine residues of DNA, lipid peroxidation, and DNA cleavage (*4*−*6*). At physiological pH (pK_a −6.8) $ONOO^-$ is protonated to form peroxynitrous acid, which decays rapidly to form a mixture of highly toxic oxidizing and nitrating species, apparently including hydroxyl radical ($^\bullet OH$) (*7*, *8*). $ONOO^-$ and its derived products have been reported to modify amino acids of proteins and induce lipid peroxidation (*9*).

For example, tyrosine is especially susceptible to $ONOO^-$-dependent nitration reactions, forming 3-nitrotyrosine that is reported as the major reaction product of proteins with $ONOO^-$ (*10*). 3-Nitrotyrosine has been detected in various diseases, including in atherosclerotic lesions of human coronary arteries. Furthermore, $ONOO^-$-modified oxidized low-density lipoprotein (oxLDL) induces endothelial apoptosis (*11*), and oxLDL may be of central importance in triggering atherosclerosis. The widespread cytotoxic effects of oxLDL are also well-known (*12*, *13*).

Since endogenous scavenging enzymes responsible for $ONOO^-$ inactivation are lacking, finding of scavengers specific to $ONOO^-$ is of considerable importance. Currently, there is an increasing interest in screening natural products for possible $ONOO^-$ scavengers. For that, studying the scavenging effect of sinapic acid (**Figure 1**) from natural products is a worthy endeavor.

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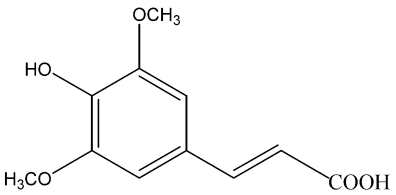

Figure 1. Structure of sinapic acid.

SA is a widely prevalent substance in the plant kingdom, such as rye (*14*), fruits, and vegetables (*15*). Although its antioxidative capacity (*14*, *15*) has been shown against other reactive oxidants, almost no information is reported about SA's action against $ONOO^-$. *Brassica juncea* is a well-known herbaceous plant, biennial or perennial, growing in China, Japan, and Korea. For the current study, SA used in this study was isolated from the ethyl acetate (EtOAc) fraction of *B. juncea*.

The purpose of this paper is to demonstrate the $ONOO^-$ scavenging activity of SA isolated from *B. juncea* and to document that SA is a strong inhibitor of the formation of serum protein nitration and LDL lipid peroxidation.

MATERIALS AND METHODS

Materials. 3-Morpholinosydnonimine hydrochloride (SIN-1), DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid), LDL, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123 (DHR 123) and $ONOO^-$ were from Molecular Probes (Eugene, OR) and Cayman Chemical Co. (Ann Arbor, MI), respectively. 4,5-Diaminofluorescein (DAF-2) was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Poly(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA), and the chemiluminescence detection system was from Amersham Life Sciences, Inc. (Arlington Heights, IL). Anti-nitrotyrosine antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep were obtained from Upstate Biotechnology (Lake Placid, NY) and Amersham (Piscataway, NJ), respectively. All other chemicals were of the highest purity available from either Sigma Chemical Co. (St. Louis, MO) or Junsei Chemical Co. (Tokyo, Japan).

Preparation of *B. juncea* Extract. The leaves of *B. juncea* were collected in August 1998 from Yosu, Chonnam Province, Korea. The powdered dried leaves of *B. juncea* were extracted by a reflux process for 3 h using methyl alcohol (MeOH). The filtrates were concentrated to dry in a vacuum at 40 °C to yield the MeOH extract. Following suspension in distilled water, the extract was further processed by partitioning with dichloromethane (CH_2Cl_2), EtOAc, EtOAc-insoluble intermediate phase (interphase), *n*-butyl alcohol (BuOH), and H_2O in sequence to get the CH_2Cl_2 fraction, EtOAc fraction, interphase fraction, BuOH fraction, and H_2O fraction, respectively. The EtOAc extract (2.5 g) was chromatographed over a silica gel column (24 × 600 mm, silica gel 60, Merck, 200 g) and eluted with CH_2Cl_2−MeOH (20:1 to 5:1) to obtain eight fractions (fractions 1−8). Combined fractions 2 and 3 (400 mg) were subjected to column chromatography over a silica gel column with CH_2Cl_2−MeOH (10:1) to give sinapic acid (20 mg), which was identified by comparing with the authentic sample purchased from Sigma.

Measurement of $ONOO^-$ Scavenging Activity. $ONOO^-$ used in the experiments was purchased from Cayman Chemical Co. or derived by SIN-1. $ONOO^-$ scavenging ability was measured by monitoring the oxidation of DHR 123 according to a modification of the method by Kooy et al. (*16*). A stock solution of DHR 123 (5 mM) purged with nitrogen was prepared in advance and stored at −20 °C. A working solution of DHR 123 (5 μM) diluted from the stock solution was placed on ice in the dark immediately prior to each experiment. The rhodamine buffer (sodium phosphate dibasic, 50 mM; sodium phosphate monobasic, 50 mM; sodium chloride, 90 mM; potassium chloride, 5 mM) including diethylenetriaminepentaacetic acid (DTPA, 5 mM) was purged with nitrogen and placed on ice before use.

The $ONOO^-$ scavenging ability was determined at room temperature by a microplate fluorescence spectrophotometer FL500 (Bio-Tek Instruments) with excitation and emission wavelengths of 485 and 530 nm, respectively. The background and final fluorescent intensities were measured 5 min after treatment with or without native $ONOO^-$ (10 μM) in 0.3 N sodium hydroxide (0.3 N NaOH) or SIN-1 (10 μM). Oxidation of DHR 123 by decomposition of SIN-1 gradually increased, whereas native $ONOO^-$ rapidly oxidized DHR 123 with its final fluorescent intensity being stable over time on the inhibition of DHR 123 oxidation by $ONOO^-$. Penicillamine was used as a positive control.

Measurement of $O_2^{\bullet -}$ Scavenging Activity. The method that measured $O_2^{\bullet -}$ scavenging activity was previously reported by Park et al. (*17*). Briefly, 2,7-Dichlorodihydrofluorescein diacetate (H_2DCFDA, 2.5 mM) mixed with esterase (1.5 units/mL) was incubated at 37 °C for 20 min and placed on ice in the dark until immediately prior to the study. Phosphate buffer (50 mM) at pH 7.4 was used. H_2DCFDA was deacetylated to nonfluorescent 2,7-dichlorodihydrofluorescein (DCFH) by esterase and subsequently oxidized to highly fluorescent 2,7-dichlorofluorescein (DCF) by $O_2^{\bullet -}$. The extent of conversion of DCFH into DCF was stoichiometrically related to the amount of $O_2^{\bullet -}$. The fluorescence intensity of oxidized DCFH was measured by using the fluorescence reader FL500 at excitation and emission wavelengths of 485 and 530 nm, respectively, for 1 h with or without the addition of menadione (50 mM) as an $O_2^{\bullet -}$ source.

Measurement of •NO Scavenging Activity. 4,5-Diaminofluorescein (DAF-2), a specific •NO indicator, selectively traps •NO between two amino groups in its molecule, yielding triazolofluorescein, which emits green fluorescence when excited at 490−495 nm (*18*). To measure •NO scavenging ability, in brief, 1 mg of DAF-2 in 0.55 mL of dimethyl sulfoxide was diluted with 50 mM phosphate buffer (pH 7.4) to $^1/_{400}$-fold. A •NO donor, sodium nitroprusside (SNP, 2 mM), and DAF-2 (3.14 μM) were added to a 96-well microplate. The fluorescence intensity was dependent on the amount of •NO trapped by DAF-2. The fluorescence signal caused by the reaction of DAF-2 with •NO was measured by the fluorescence spectrometer FL500 at excitation and emission wavelengths of 495 and 515 nm after 10 min.

Measurement of •OH Scavenging Activity. The method that measured •OH scavenging activity was previously reported by Halliwell et al. (*19*). A 96-well microplate was used. *B. juncea* extracts (or components) of 10% v/v EtOH solution (10 μL) were added to 1.25 mM H_2O_2 and 0.2 mM $FeSO_4$ and incubated at 37 °C for 5 min. Then esterase-treated 2 μM H_2DCFDA was added, and the final volume is 250 μL/well. Changes in fluorescence were monitored by the fluorescence spectrophotometer FL500 at an excitation wavelength of 485 nm and an emission wavelength of 530 nm for 40 min.

Reaction of SA with $ONOO^-$. The reaction between SA and $ONOO^-$ was investigated. $ONOO^-$ (200 μM) was reacted with SA (100 μM) in 50 mM phosphate buffer at pH 7.0 at room temperature for 10 min, followed by a spectrophotometric scan with a UV/visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech) from 190 to 600 nm.

Inhibition of $ONOO^-$-Mediated Tyrosine Nitration by SA. The ability of SA to inhibit the formation of 3-nitrotyrosine was quantitated as the index of SA's inhibition of tyrosine nitration utilizing spectrophotometric analysis and western blot analysis.

(1) Spectrophotometric Analysis of Nitrated Proteins. Tyrosine (200 μM) was reacted with $ONOO^-$ (200 μM) in the presence of varying concentrations of SA (0−100 μM). The formations of 3-nitrotyrosine were determined with a UV/visible spectrophotometer. Tyrosine was monitored at 275 nm while 3-nitrotyrosine formations were monitored at 430 nm. The disappearance of 3-nitrotyrosine peaks at 430 nm in the presence of SA was taken as an indication of the inhibition by SA.

(2) Western Blotting Analysis. A 2.5 μL aliquot of SA dissolved in 10% v/v EtOH was added to 95 μL of BSA (0.5 mg of protein/mL) or LDL (1 mg of protein/mL). The mixed samples were incubated with shaking at 20 °C for 1 h. After 1 h incubation, 2.5 μL of $ONOO^-$ (100 μM) in 0.3 N NaOH was added. The samples were then incubated for 30 min at 20 °C with shaking.

The samples prepared in gel loading buffer [pH 6.8; 0.125 M tris(hydroxymethyl)aminomethane (Tris), 4% mass per volume (m/v) sodium dodecyl sulfate (SDS), 20% m/v glycerol, 10% m/v 2-mer-

Table 1. Peroxynitrite Scavenging Activities of *B. juncea* Fractions

fraction	IC_{50} (μg/mL)	fraction	IC_{50} (μg/mL)
EtOAc	4.90 ± 0.48	CH_2Cl_2 in MeOH	43.28 ± 4.43
BuOH	9.51 ± 0.52	CH_2Cl_2 in hexane	≫100
interphase	10.20 ± 0.70	penicillamine[a]	0.44 ± 0.00
H_2O	33.39 ± 1.13		

[a] Used as a positive control. Abbreviations: IC_{50}, 50% inhibition concentration; EtOAc, ethyl acetate; BuOH, *n*-butyl alcohol; CH_2Cl_2, dichloromethane; MeOH, methyl alcohol; interphase, EtOAc-insoluble intermediated phase.

captoethanol, and 0.2% m/v bromophenol blue] in a ratio of 1:1 were boiled for 5 min. Twenty microliters of each sample was separated on an SDS−polyacrylamide minigel (10% for BSA, 6% for LDL) at 100 V and transferred to a poly(vinylidene fluoride) membrane at 100 V for 1.5 h in a wet transfer system (Bio-Rad, Hercules, CA).

The membrane was immediately placed into a blocking solution (10% m/v skim milk powder in TBS−Tween buffer containing 10 mM Tris, 100 mM NaCl, and 0.1 mM Tween-20, pH 7.5) at 4 °C overnight. The membrane was washed in TBS−Tween buffer for 30 min and then incubated with a monoclonal anti-nitrotyrosine antibody (0.5% m/v skim milk, diluted 1:2000 in TBS−Tween buffer) at room temperature for 2 h. After four 10 min washings in TBS−Tween buffer, the membrane was reacted with a horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep (0.1% m/v skim milk, diluted 1:2000 in TBS−Tween buffer) at room temperature for 2 h. After four 10 min washings in TBS−Tween buffer, antibody labeling was detected using enhanced chemiluminescence and exposed to radiographic film. A prestained blue protein marker was used for molecular weight determination.

Lipid Peroxidation Assay. The method that measures the oxidation of LDL by SIN-1 has been previously described (*20*). Briefly, LDL at a concentration of 2 mg/mL was oxidized by 800 μM SIN-1 to obtain final concentrations of 1 mg/mL LDL and 200 μM SIN-1 in Dulbecco's phosphate-buffered saline for 4 h incubation at 37 °C. The amounts of free malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) were used as markers for lipid peroxidation using a lipid peroxidation assay kit (Calbiochem, San Diego, CA) according to manufacturer's instructions. This assay is based on the reaction of MDA and 4-HNE with the chromogenic reagent *N*-methyl-2-phenylindole, forming a stable chromophone, which absorbs at 586 nm. Following incubation with SIN-1 and SA, LDL samples as well as MDA standards were incubated with the chromogen at 45 °C. The MDA content was expressed as micromolar MDA generated per milligram of LDL protein.

Statistical Analysis. All results are expressed as mean ± SD of three separate experiments unless stated otherwise. Statistical analysis was determined by Student's *t*-test (Statworks); $p < 0.05$ was considered to be statistically significant.

RESULTS

$ONOO^-$ Scavenging Activities of *B. juncea* Extracts. The oxidation of DHR 123 to fluorescent rhodamine 123 by native $ONOO^-$ or SIN-1-derived $ONOO^-$ was determined in the presence of the *B. juncea* extracts at increasing concentrations. Then a 50% inhibition concentration (IC_{50}) was calculated. The comparative data on the potency of the inhibition of DHR 123 oxidation by $ONOO^-$ are as shown in **Table 1**. The $ONOO^-$ scavenging activity of *B. juncea* extracts was highest in the EtOAc fraction (4.90 ± 0.48 μg/mL), followed by the BuOH fraction (9.51 ± 0.52 μg/mL) and the interphase fraction (10.20 ± 0.70 μg/mL).

$ONOO^-$ Scavenging Activity of SA Isolated from *B. juncea*. The $ONOO^-$scavenging ability of SA was investigated using DHR 123. The results showed that SA owns a more potent scavenging activity (IC_{50} = 0.58 ± 0.12 mM) compared to penicillamine (IC_{50} = 2.93 ± 0.05 mM), which was used as a reference compound. To simulate physiological conditions with high CO_2 concentrations in vivo, Na_2CO_3 was used as a CO_2 donor in this study. When $ONOO^-$ scavenging activity was measured in the presence of 25 mM Na_2CO_3, the high scavenging activity of SA (IC_{50} of 1.10 ± 0.11 mM) was exhibited. In the case of the scavenging activity of $ONOO^-$ that was generated from SIN-1 reactions, SA showed even a higher ability than penicillamine as shown in **Table 2**. Their IC_{50} values are 1.58 ± 0.12 and 3.80 ± 0.15 mM for SA and penicillamine, respectively.

Table 2. Peroxynitrite ($ONOO^-$) Scavenging Activity of Sinapic Aicd Isolated from *B. juncea*

	IC_{50} value (μM)		
	native $ONOO^-$		
name	without Na_2CO_3	with 25 mM Na_2CO_3	SIN-1 derived $ONOO^-$
sinapic acid	0.58 ± 0.12	1.10 ± 0.11	1.58 ± 0.12
penicillamine[a]	2.93 ± 0.05	1.10 ± 0.49	3.80 ± 0.15

[a] Used as a positive control. IC_{50}: 50% inhibition concentration.

Table 3. IC_{50} (μM) of Sinapic Acid on Related Free Radical Scavenging Activities

	•NO	$O_2^{\bullet-}$	•OH
sinapic acid	3.99 ± 0.06	17.98 ± 1.60	3.80 ± 0.15
carboxy-PTIO[a]	16.73 ± 2.28		
trolox[a]		7.24 ± 0.25	
vitamin C[a]			4.56 ± 0.83

[a] Used as a positive control. Abbreviations: •NO, nitro oxide radical; $O_2^{\bullet-}$, superoxide; •OH, hydroxyl radical.

Scavenging Ability of SA against Other Reactive Species. **Table 3** presents IC_{50} values of SA for $O_2^{\bullet-}$, •NO, and •OH scavenging activities. Because $ONOO^-$ can be formed from a reaction of $O_2^{\bullet-}$ with •NO, it is interesting to compare the active component's ability for the $ONOO^-$ and $O_2^{\bullet-}$ and •NO scavenging activities. In comparison with a •NO scavenging standard carboxy-PTIO (IC_{50} = 16.73 ± 2.28 mM), SA had a higher activity (IC_{50} = 3.99 ± 0.06 mM).

In addition, SA's inhibitory action was considerately strong (IC_{50} = 17.98 ± 1.60 mM) in $O_2^{\bullet-}$ scavenging activity, compared with a well-known antioxidant, trolox (IC_{50} = 7.24 ± 0.25 mM). The data also demonstrated that SA (IC_{50} = 3.80 ± 0.15 mM) can scavenge •OH radical as effective as antioxidant vitamin C (IC_{50} = 4.56 ± 0.83 mM).

Reaction of SA with $ONOO^-$. To determine the mechanism of SA's $ONOO^-$ scavenging activity, the reaction process of SA with $ONOO^-$ was investigated. The spectrum changes at 430 nm were monitored spectrophotometrically to ascertain whether SA undergoes a nitration process when reacting with $ONOO^-$. The interaction of SA with $ONOO^-$ produced no spectral changes at 430 nm (**Figure 2B**) as compared with original spectrum graph in **Figure 2A**, indicating no nitration formation. Interestingly, the appearance of a new peak at 340 nm (**Figure 2B**) was detected, implying an unidentified by-product formed from the reaction of SA and $ONOO^-$.

Effect of SA on $ONOO^-$-Mediated 3-Nitrotyrosine Formation. Tyrosine undergoes nitration to form 3-nitrotyrosine when exposed to $ONOO^-$ at pH 7, showing a peak at 430 nm. In the present study, it was found that exposure of tyrosine (200 μM) to increasing concentrations of $ONOO^-$ (0−1000 μM) resulted in an increased production of 3-nitrotyrosine (data not shown).

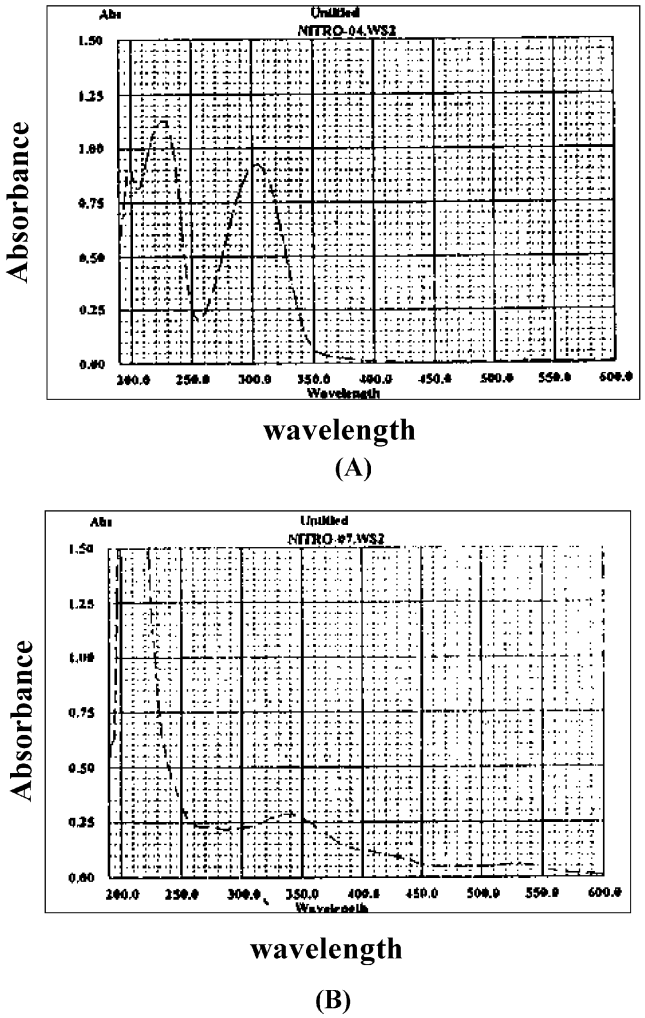

Figure 2. Interaction of sinapic acid (SA) with peroxynitrite ($ONOO^-$). SA (100 μM) was incubated without (A) or with (B) $ONOO^-$ (200 μM) in 50 mM phosphate buffer at pH 7.0 at room temperature for 10 min, followed by a spectrophotometric scan from 190 to 600 nm.

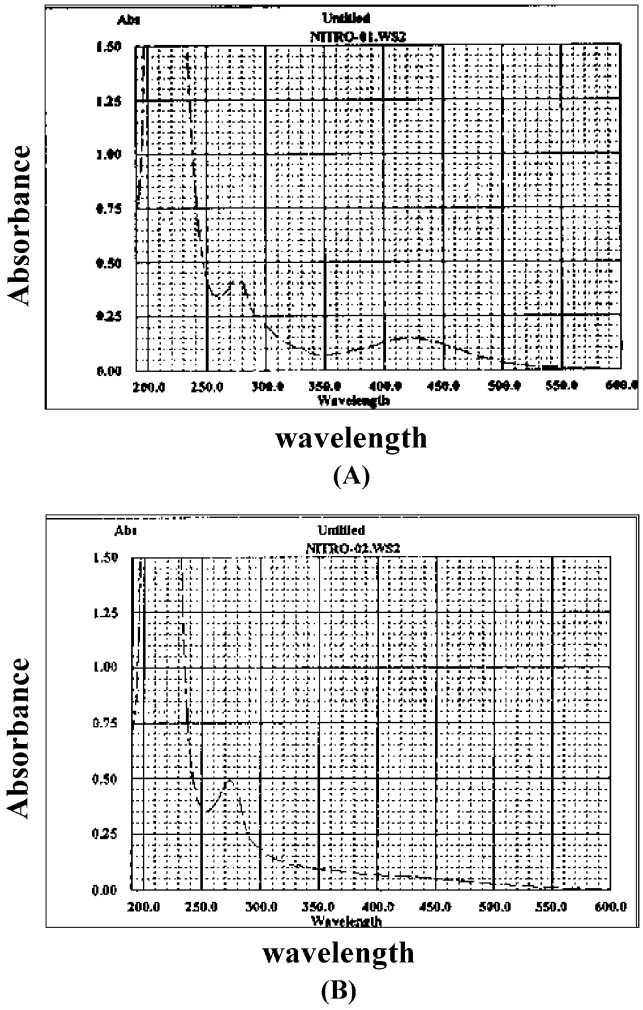

Figure 3. Effect of sinapic acid (SA) with peroxynitrite- ($ONOO^-$-) mediated 3-nitrotyrosine. Tyrosine (200 μM) was incubated without (A) or with (B) SA prior to the addition of $ONOO^-$. Each mixed solution was incubated at 37 °C with shaking for 1 h and scanned between 190 and 600 nm with spectrophotometric analysis. The spectrum of the peak displayed at 430 nm reflects the formation of 3-nitrotyrosine.

Nitration of tyrosine can be easily detected by color change from colorless to the characteristic yellow color when tyrosine and $ONOO^-$ are mixed. Once nitrotyrosine was formed at pH 7, the absorbance was maximal at the 420−440 nm range, as shown in **Figure 3A**. Incubation of SA with tyrosine prior to the addition of $ONOO^-$ resulted in the disappearance of the nitrotyrosine peak at 430 nm (**Figure 3B**), implying that SA inhibited the formation of 3-nitrotyrosine. Although data are not presented, the extent of this inhibition was dose-dependent.

Effect of SA on Nitration of BSA and LDL. Using anti-3-nitrotyrosine antibody by western blot, we analyzed the ability of SA to suppress $ONOO^-$-mediated tyrosine nitration in common biological materials, such as BSA and LDL. For this purpose, SA was preincubated with BSA or LDL prior to the addition of 100 μM $ONOO^-$ and checked for the formation of 3-nitrotyrosine. The results revealed that preincubation of SA at concentrations of 1, 5, 25, 50, and 100 μM attenuated the nitration of BSA or LDL in a dose-dependent manner, as shown in **Figure 4**.

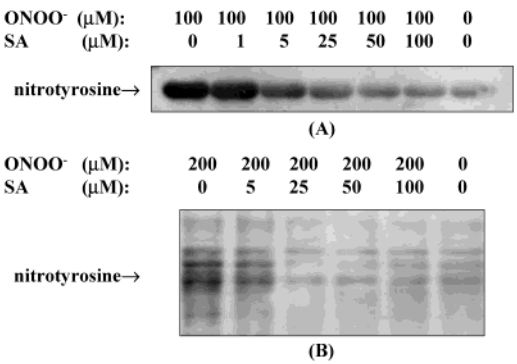

Figure 4. Effect of sinapic acid (SA) on the nitration of BSA and LDL by peroxynitrite ($ONOO^-$). SA was added to BSA (A) or LDL (B). The reaction samples were incubated with shaking at 20 °C for 1 h. After $ONOO^-$ was added, all samples were further incubated with shaking at 20 °C for 30 min. Abbreviations: BSA, bovine serum albumin; LDL, low-density lipoprotein.

Effect of SA on $ONOO^-$-Induced Lipid Peroxidation. When human plasma LDL was incubated with SIN-1 (1 mM), a significant increase in the products of lipid peroxidation was observed (291.87 ± 18.21%), as shown in **Figure 5**. The relative effectiveness of SA on a decreased LDL oxidation is shown in **Table 4**. SIN-1 was added to LDL (1 mg of protein/mL) after pretreatment with various concentrations of SA or trolox in the range of 5, 10, 20, 50, and 100 μM. The results (**Table 4**) show that SA was able to prevent the modification of LDL by $ONOO^-$ in a dose-dependent manner.

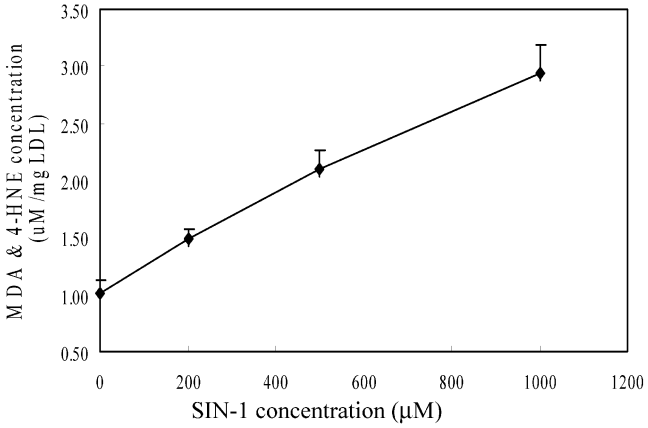

Figure 5. SIN-1-induced MDA and 4-HNE formations in LDL. LDL (1 mg/mL) was incubated with variable concentrations of SIN-1 for 4 h at 37 °C in a water bath. MDA levels are expressed as the concentration produced in micromolar per milligram of LDL protein. The mean ± SE of duplicate samples is shown. The results were confirmed by at least three more independent experiments. Abbreviations: SIN-1, 3-morpholinosydnonimine hydrochloride; MDA, malondialdehyde; 4-HNE, 4-hydroxyalkenals; LDL, low-density lipoprotein.

Table 4. Reduction Percentage of Sinapic Acid Decrease in SIN-1-Induced LDL Peroxidation[a]

sinapic acid concn (μM)	% reduction	sinapic acid concn (μM)	% reduction
5	38.72 ± 1.68	50	63.69 ± 2.28
10	53.74 ± 1.01	100	76.39 ± 3.47
20	59.67 ± 2.11	trolox 100	61.22 ± 3.04

[a] LDL (1 mg/mL in PBS) was incubated at 37 °C for 4 h with 1 mM SIN-1 in the presence of 5, 10, 20, 50, and 100 μM sinapic acid. Abbreviations: SIN-1, 3-morpholinosydnonimine hydrochloride; LDL, low-density lipoprotein.

DISCUSSION

$ONOO^-$ is a potent mediator in the inflammatory process and atherogenesis with its strong oxidizing property toward biological molecules (*21*). As a member of reactive species, $ONOO^-$ has been implicated in several major chronic diseases such as Alzheimer's disease, rheumatoid arthritis, cancer, and atherosclerosis (*22*).

The toxicity of $ONOO^-$ can be attributed to nitration of tyrosine and tryptophan residues and subsequent alterations of their functionalities (*23*). Plant food-derived antioxidants and active principles such as flavonoids, hydroxycinnamates (ferulic acid, chlorogenic acids, vanillin, etc.), β-carotene and other carotenoids, vitamin E, vitamin C, rosemary, sage, and tea are important dietary antioxidant substances (*24*) that may provide efficient $ONOO^-$ scavengers. SA, a component widely prevalent in the plant kingdom, has been suggested to possess an antioxidant property (*15*, *25*).

In the current research, SA isolated from the plant *B. juncea* was shown to have a strong scavenging activity against $ONOO^-$ (**Table 2**). In this study, well-known $ONOO^-$, $^{\bullet}NO$, and $O_2^{\bullet-}$ scavenging agents penicillamine (*26*), carboxy-PTIO (*27*), and trolox (*28*) were respectively used for the comparative purposes in this study. The important finding from the present study is that SA isolated from *B. juncea* not only directly scavenged $ONOO^-$ avidly but also inhibited $O_2^{\bullet-}$ and $^{\bullet}NO$ radical formation (**Table 3**). On the basis of the SA's structural similarity with other known antioxidants containing hydroxyl groups, it is likely that the hydroxyl group of the SA structure may be responsible for the $ONOO^-$ scavenging activity (**Figure 1**).

Under the physiological conditions, the reaction of $ONOO^-$ with bicarbonate is predominant, and the rate constant of reaction of CO_2 with $ONOO^-$ is large (pH-independent, $k = 5.8 \times 10^4\ M^{-1}\ s^{-1}$) (*28*). Thus, the reactivity of the putative scavengers for $ONOO^-$ should be able to match or exceed that of bicarbonate (*29*). It has been reported that bicarbonate (25 mM) significantly weakens the ability of several phenolic compounds, such as caffeic acid, *o*- and *p*-coumaric acid, gallic acid, and ferulic acid (*30*). To look into the SA case, the present study evaluated $ONOO^-$ scavenging activity in the presence of Na_2CO_3. It was found that, under this condition, SA still showed high $ONOO^-$ scavenging activity as $IC_{50} = 1.10 \pm 0.11$ μM (**Table 2**). Another important occurrence under physiological conditions is the formation of highly reactive $^{\bullet}OH$ due to the decomposition of $ONOO^-$ (*5*). In our study, we detected $^{\bullet}OH$ scavenging activity of SA. Compared with vitamin C ($IC_{50} = 4.56 \pm 0.83$ μM), as a positive control, SA showed a considerable $^{\bullet}OH$ scavenging activity ($IC_{50} = 3.80 \pm 0.15$ μM). These data strongly indicate that SA's antioxidant capacity is diverse, yet highly efficient.

Although the detailed mechanism of the $ONOO^-$ scavenging action is not yet clearly known, so far, two possible pathways, nitration or electron donation, have been proposed for the interaction of a phenolic $ONOO^-$ scavenger with $ONOO^-$ (*31*). Phenolic components in the case of monohydroxy-related structures such as *p*-coumaric acid and ferulic acid can inhibit $ONOO^-$-mediated nitration of tyrosine by acting as alternative substrates for nitration or by reducing reactive nitrogen species as has been demonstrated for catechol structures such as caffeic acid (*32*).

In the current study, to determine whether SA can undergo the nitration reaction, SA was exposed to $ONOO^-$. The addition of $ONOO^-$ revealed no spectral changes in the visible region, which suggested that nitration of the aromatic ring has not occurred. In addition, preincubation of tyrosine with SA before $ONOO^-$ addition caused a decreased peak at 430 nm of nitrotyrosine, which is further evidence supporting the possibility of an electron donation reaction between SA and $ONOO^-$.

There was, however, a change in the spectra of the samples in the UV region during reaction of SA and $ONOO^-$. As shown in **Figure 2B**, a peak appeared at around 340 nm, indicating a possibility of a new unidentified compound produced. A similar finding had been reported by Niwa et al. (*33*), who used a HPLC system to isolated this new compound from reaction media of SA with $ONOO^-$ and suggested a one-electron oxidation may occur during the reaction.

Furthermore, the possible interruption of SA on the interaction between tyrosine and $ONOO^-$ was evident by showing on the decreased peak of 3-nitrotyrosine (**Figure 3**). This result suggested that SA directly blocked the formation of 3-nitrotyrosine by the action of $ONOO^-$ and possibly reduced the availability of $ONOO^-$.

The inhibitory action of SA was further tested with more physiological substances such as BSA or LDL in the current study. BSA, the most abundant protein in plasma for the maintenance of colloid osmotic pressure and the transport of different legends, and LDL of human plasma were used as models for $ONOO^-$-induced protein nitration. Protein modification by tyrosine nitration due to $ONOO^-$ interferes with phosphorylation/dephosphorylation signaling pathways and altered enzyme functions (*23*). In the present study, evidence from western blot analysis indicated that SA even at concentra-

tion as low as 5 mM could significantly reduce nitrotyrosine present in BSA and LDL. With the increase of SA concentration, the nitration of BSA and LDL decreased, which is further suggestive of the putative in vivo anti-$ONOO^-$ action.

$ONOO^-$ is closely implicated as an oxidant for LDL in atherogenesis. Exposure to $ONOO^-$ resulted in rapid (<1 min) and time-dependent oxidation of LDL's lipids and protein (*34*). $ONOO^-$, formed in the arterial wall from the reaction of $O_2^{\bullet-}$ with $^{\bullet}NO$, can initiate lipid peroxidation and oxidative damage to LDL and leave arteries susceptible to chronic inflammation, causing modification of arterial gene expression and promotion of lesion development (*35*). Because antioxidant defenses are limited in the microenvironment of the cell or within LDL molecules, the oxidation process continues to progress (*36*). Therefore, protection against excessive $ONOO^-$ becomes necessary.

There have been several reports that demonstrate SIN-1-derived $ONOO^-$ leads to LDL oxidation (*20*, *37*, *38*). It was also suggested that $ONOO^-$ modification of LDL, using SIN-1 as the resource of $ONOO^-$, might represent a more biologically relevant model for in vitro studies of oxLDL (*20*). In our study, incubation of LDL of human plasma with the $ONOO^-$ donor SIN-1 resulted in the formation of the lipid peroxidation products, MDA and 4-HNE (**Figure 5**), which were dependent on the concentration of SIN-1. Incubated SIN-1 alone did not absorb at 586 nm. When added SA to SIN-1 induced oxLDL, MDA and 4-HNE formations were inhibited in a dose-dependent manner (**Table 4**), and interestingly, SA seems to be more efficient than trolox (% reduction of 76.39 ± 3.47 vs 61.22 ± 3.04 at 100 μM).

To conclude the discussion, the present study demonstrated that the active component SA, isolated from *B. juncea*, can scavenge $ONOO^-$ as well as SIN-1-induced $ONOO^-$ efficiently. SA led to a decrease of $ONOO^-$-mediated nitration of tyrosine through electron donation, and it also showed significant inhibition ability on nitration of BSA and LDL by $ONOO^-$ in a dose-dependent manner. SA can limit SIN-1-induced LDL lipid peroxidation products equally well. In summary, the present results produced reliable new information that SA is an efficient $ONOO^-$ scavenger that has a potential to be an effective $ONOO^-$ scavenger for the prevention of the $ONOO^-$-involved diseases.

ABBREVIATIONS USED

BSA, bovine serum albumin; BuOH, butyl alcohol; CH_2Cl_2, dichloromethane; EtOAc, ethyl acetate; 4-HNE, 4-hydroxyalkenals; LDL, low-density lipoprotein; MDA, malondialdehyde; $^{\bullet}NO$, nitro oxide radical; $O_2^{\bullet-}$, superoxide; $^{\bullet}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; SA, sinapic acid; SIN-1, 3-morpholinosydnonimine hydrochloride.

LITERATURE CITED

(1) Hughes, M. N.; Nicklin, H. G. The chemistry of peroxynitrites. Part I. The kinetics of decomposition of peroxynitrous acid. *J. Chem. Soc. A* **1968**, 450−453.

(2) Maeda, H. Deleterious pathogenic mechanism involving host response in influenza virus infection in mice. *Nippon Rinsho* **1997**, *55*, 2676−2681.

(3) Banan, A.; Fields, J. Z.; Decker, H.; Zhang, Y.; Keshavarzian, A. Nitric oxide and its metabolites mediate ethanol-induced microtubule disruption and intestinal barrier dysfunction. *J. Pharmacol. Exp. Ther.* **2000**, *294*, 997−1008.

(4) Akaike, T.; Suga, M.; Maeda, H. Free radicals in viral pathogenesis: molecular mechanisms involving superoxide and NO. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 64−73.

(5) Elliott, S. J.; Lacey, D. J.; Chilian, W. M.; Brzezinska, A. K. Peroxynitrite is a contractile agonist of cerebral artery smooth muscle cells. *Am. J. Physiol.* **1998**, *275*, 1585−1591.

(6) Viner, R. I.; Ferrington, D. A.; Williams, T. D.; Bigelow, D. J.; Schoneich, C. Protein modification during biological aging: selective tyrosine nitration of SERCA2a isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase in skeletal muscle. *J. Biochem.* **1999**, *340*, 657−669.

(7) Beckman, J. S.; Chen, J.; Ischiropoulos, H.; Crow, J. P. Oxidative chemistry of peroxynitrite. *Methods Enzymol.* **1994**, *233*, 229−240.

(8) Van der Vliet, A.; O'Neill, C. A.; Halliwell, B.; Cross, C. E.; Kaur, H. Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite. Evidence for hydroxyl radical production from peroxynitrite. *FEBS Lett.* **1994**, *339*, 89−92.

(9) Whiteman, M.; Halliwell, B. Protection against peroxynitrite-dependent tyrosine nitration and alpha 1-antiproteinase inactivation by ascorbic acid. A comparison with other biological antioxidants. *Free Radical Res.* **1996**, *25*, 275−283.

(10) Ischiropoulos, H.; Beckman, J. S.; Crow, J. P.; Ye, Y. Z.; Royall, J. A.; Kooy, N. W. Detection of peroxynitrite. *Methods* **1995**, *7*, 109−115.

(11) Kotamraju, S.; Hogg, N.; Joseph, J.; Keefer, L. K.; Kalyanaraman, B. Inhibition of oxidized low-density lipoprotein-induced apoptosis in endothelial cells by nitric oxide. Peroxyl radical scavenging as an antiapoptotic mechanism. *J. Biol. Chem.* **2001**, *276*, 17316−17323.

(12) Darley-Usmar, V. M.; Hogg, N.; O'Leary, V. J.; Wilson, M. T.; Moncada, S. The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low-density lipoprotein. *Free Radical Res. Commun.* **1992**, *17*, 9−20.

(13) Graham, A.; Hogg, N.; Kalyanaraman, B.; O'Leary, V.; Darley-Usmar, V. M.; Moncada, S. Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett.* **1993**, *330*, 181−185.

(14) Andreasen, M. F.; Landbo, A. K.; Christensen, L. P.; Hansen, A.; Meyer, A. S. Antioxidant effect of phenolic rye (*Secale cereale* L.) extracts, monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoproteins. *J. Agric. Food Chem.* **2001**, *49*, 4090−4096.

(15) Lu, C.; Yao, S.; Lin, N. Studies on reactions of oxidizing sulfur-sulfur three-electron-bond complexes and reducing alpha-amino radicals derived from OH reaction with methionine in aqueous solution. *Biochim. Biophys. Acta* **2001**, *16*, 89−96.

(16) Kooy, N. W.; Royall, J. A.; Ischiropulos, H.; Beckman, J. S. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radical Biol. Med.* **1994**, *16*, 149−156.

(17) Park, H. J.; Chung, H. Y.; Kim, J.; Choi, J. S. Antioxidant activity of 2,3,6-tribromo-4,5-dihydroxy-benzylmethyl ether from *Symphyocladia latiuscula*. *J. Fish Sci. Technol.* **1999**, *2*, 1−7.

(18) Nagata, N.; Momose, K.; Ishida, Y. Inhibitory effects of catecholamines and anti-oxidants on the fluorescence reaction of 4,5-diaminofluorescein, DAF-2, a novel indicator of nitric oxide. *J. Biochem. (Tokyo)* **1999**, *125*, 658−661.

(19) Halliwell, B.; Gutteridge, J. M. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.* **1990**, *186*, 1−85.

(20) Guy, R. A.; Maguire, G. F.; Crandal, I. L.; Connelly, P. W.; Kain, K. C. Characterization of peroxynitrite-oxidized low-density lipoprotein binding to human CD36. *Arteriosclerosis* **2001**, *155*, 19−28.

(21) Podrez, E. A.; Schmitt, D.; Hoff, H. F.; Hazen, S. L. Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. *J. Clin. Invest.* **1999**, *103*, 1547−1560.

(22) Beckman, J. S.; Koppenol, W. H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* **1996**, *271*, C1424−C1437.

(23) Li, X.; De Sarno, P.; Song, L.; Beckman, J. S.; Jope, R. S. Peroxynitrite modulates tyrosine phosphorylation and phosphoinositide signaling in human neuroblastoma SH−SY5Y cells: attenuated effects in human 1321N1 astrocytoma cells. *Biochem. J.* **1998**, *331*, 599−606.

(24) Sies, H.; Klotz, L. O.; Sharov, V. S.; Assmann, A.; Briviba, K. Protection against peroxynitrite by selenoproteins. *Z. Naturforsch., C* **1998**, *53*, 228−232.

(25) Chalas, J.; Claise, C.; Edeas, M.; Messaoudi, C.; Vergnes, L.; Abella, A.; Lindenbaum, A. Effect of ethyl esterification of phenolic acids on low-density lipoprotein oxidation. *Biomed. Pharmacother.* **2001**, *55*, 54−60.

(26) Pannala, A. S.; Rice-Evans, C.; Halliwell, B.; Singh, S. Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. *Biochem. Biophys. Res. Commun.* **1997**, *232*, 164−168.

(27) Yoshida, M.; Akaike, T.; Goto, S.; Takahashi, W.; Inadome, A.; Yono, M.; Seshita, H.; Maeda, H.; Ueda, S. Effect of the NO scavenging carboy-ptio on endothelium-dependent vasorelaxation of various blood vessels from rabbits. *Life Sci.* **1998**, *62*, 203−211.

(28) Satoh, K.; Kadofuku, T.; Sakagami, H. Effect of trolox, a synthetic analog of α-tocopherol, on cytotoxicity induced by UV irradiation and antioxidants. *Anticancer Res.* **1997**, *17*, 2459−2463.

(29) Squadrito, G. L.; Pryor, W. A. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radical Biol. Med.* **1998**, *25*, 392−403.

(30) Ketsawatsakul, U.; Whiteman, M.; Halliwell, B. A reevaluation of the peroxynitrite scavenging activity of some dietary phenolics. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 692−699.

(31) Pannala, A.; Razaq, R.; Halliwell, B.; Singh, S.; Rice-Evans, C. A. Inhibition of peroxynitrite dependent nitration by hydoxycinnamates: nitration or electron donation? *Free Radical Biol. Med.* **1998**, *24*, 594−606.

(32) Kerry, N.; Rice-Evans, C. Inhibition of peroxynitrite-mediated oxidation of dopamine by flavonoid and phenolic antioxidants and their structural relationships. *J. Neurochem.* **1999**, *73*, 247−253.

(33) Niwa, T.; Doi, U.; Kato, Y.; Osawa, T. Inhibitory mechanism of sinapinic acid against peroxynitrite-mediated tyrosine nitration of protein in vitro. *FEBS Lett.* **1999**, *459*, 43−46.

(34) Thomas, S. R.; Davies, M. J.; Stocker, R. Oxidation and antioxidation of human low-density lipoprotein and plasma exposed to 3-morpholinosydnonimine and reagent peroxynitrite. *Chem. Res. Toxicol.* **1998**, *11*, 484−494.

(35) Yla-Herttuala, S.; Luoma, J.; Kallionpaa, H.; Laukkanen, M.; Lehtolainen, P.; Viita, H. Pathogenesis of atherosclerosis. *Maturitas* **1996**, *23*, 47−49.

(36) Hajjar, D. P.; Haberland, M. E. Lipoprotein trafficking in vascular cells. Molecular Trojan horses and cellular saboteurs. *J Biol. Chem.* **1997**, *272*, 22975−22978.

(37) Van der Veen, R. C.; Roberts, L. J. Contracting roles for nitric oxide and peroxynitrite in the peroxidation of myelin lipids. *J. Neuroimmunol.* **1999**, *95*, 1−7.

(38) Kotamraju, S.; Hogg, N.; Joseph, J.; Keefer, L. K.; Kalyanaraman, B. Inhibition of oxidized low-density lipoprotein-induced apoptosis in endothelial cells by nitric oxide. Peroxylradical scavenging as an antiapoptotic mechanism. *J. Biol. Chem.* **2001**, *276*, 17316−17323.

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