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Tanshinone II-A Inhibits Low Density Lipoprotein Oxidation *In Vitro*

XI-LIN NIU[a], KOHJI ICHIMORI[a], XIA YANG[a], YUKI HIROTA[a], KIYOTAKA HOSHIAI[a], MIN LI[b] and HIROE NAKAZAWA[a,*]

[a]*Department of Physiology, School of Medicine, Tokai University, Bohseidai Isehara 259-1193, Kanagawa, Japan;*
[b]*Guangzhou College of Traditional Chinese Medicine, P.R. China*

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Tanshinone II-A (TSII-A) is a major component of Salvia miltorrhiza Bunge which has long been used for preventing and ameliorating anginal pain in China. However the effect of TSII-A on low density lipoprotein (LDL) oxidation has not been studied. The present study was performed to investigate the effects of TSII-A on LDL oxidation using four oxidizing systems, including copper-, peroxyl radical- and peroxynitrite-initiated and macrophage-mediated LDL oxidation. LDL oxidation was measured in terms of formation of thiobarbituric acid-reactive substances (TBARS), relative electrophoretic mobility (REM) on agarose gel and lag time. In all four systems, TSII-A has apparent antioxidative effects against LDL oxidation, as evidenced by its dose-dependent inhibition of TBARS formation, prolongation of lag time and suppression of increased REM.

Regarding the mechanism underlying its antioxidative effect, TSII-A neither scavenged superoxide nor peroxynitrite. It also did not chelate copper. But it has mild peroxyl radical scavenging activity. The direct binding to LDL particles and conformational change of LDL structure by TSII-A were suggested, because it increased negative charge of LDL which was shown by increased REM on agarose gel. In conclusion, TSII-A is an effective antioxidant against LDL oxidation *in vitro*. The underlying mechanism appears to be related to its peroxyl radical scavenging and LDL binding activity.

Keywords: Tanshinone, antioxidant, low density lipoprotein, oxidation, free radical

Abbreviations: TSII-A, tanshinone II-A; BHT, butylated hydroxytoluene; SOD, superoxide dismutase; TBA, thiobarbituric acid; DMEM, Dulbecco's modified Eagle's medium; DHR, Dihydrorhodamine 123; HBSS, Hank's balanced salt solution; AAPH, 2,2-azobis(2-amidinopropane) dihydrochloride; $ONOO^-$, peroxynitrite; PBS, phosphate buffered saline; MeO–AMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); DPPD, N,N′-diphenyl-p-phenylene-diamine; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; DPBQ, N,N′-diphenyl-p-benzoquinone diimine; REM, relative electrophoretic mobility

INTRODUCTION

It has been well documented that oxidatively modified low density lipoprotein (ox-LDL) is present in atherosclerotic lesions and exhibits a wide range of atherogenic properties. For example, ox-LDL is chemotactic for circulating monocytes and stimulates the adhesion of monocyte to

*Corresponding author. Tel.: 81-463-93-1121, ext. 2532. Fax: 81-463-93-6684. E-mail: nakazawa@is.icc.u-tokai.ac.jp.

endothelial cells.[1,2] It can be taken up readily by macrophages, turning them into foam cells.[3,4] Furthermore, ox-LDL modulates activation of NFκB and alters arterial vasomotor properties.[5–8] Increasing evidence indicates that agents which could inhibit the oxidation of LDL are beneficial in preventing the development of atherosclerosis.[9,10]

Tanshinone II-A (TSII-A) is a derivative of phenanthrenequinone, having the molecular formula of $C_{19}O_3H_{20}$ (Figure 1). It was purified from Salvia miltorrhiza Bunge, a traditional medicine in China, which has long been used for preventing and ameliorating anginal pain in China. Recently, carbon radical scavenging activity of TSII-A was shown in Fe^{2+}-induced peroxidation of myocardial mitochondrial membrane.[11] TSII-A was also shown to have protective effects on ischemia–reperfusion injury in hearts and on lipid peroxidation-induced DNA damage in liver cells.[12,13] However, the effect of TSII-A on LDL oxidation has not been examined yet.

The present study was designed to investigate possible antioxidative properties of TSII-A on human LDL oxidation *in vitro* and to elucidate the mechanisms involved in such phenomena. In the evaluation of the possible antioxidative effects of TSII-A on human LDL oxidation, we used four different oxidative systems such as copper-, peroxyl radical-, and macrophage-induced LDL oxidation systems. We also observed the effect of TSII-A on peroxynitrite ($ONOO^-$)-induced LDL oxidation, since $ONOO^-$ has recently been shown to be involved in the LDL oxidation *in vivo*.[14]

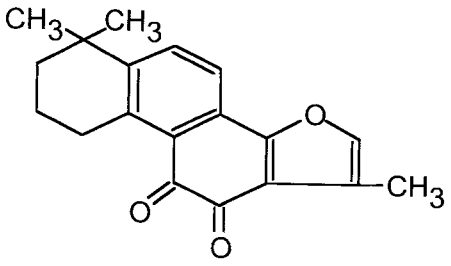

FIGURE 1 The structure of Tanshinone II-A.

MATERIALS AND METHODS

Materials

Butylated hydroxytoluene (BHT), superoxide dismutase (SOD), cytochrome *c*, thiobarbituric acid (TBA) and Dulbecco's modified Eagle's medium (DMEM, Catalog No. D-1152) were purchased from Sigma Chemical Co. (St. Louis, MO). Catalase and xanthine oxidase were obtained from Boehringer Mannheim Gmbh (Germany). Dihydrorhodamine 123 (DHR) was obtained from Molecular Probes, Inc. (Eugene OR). Ham's F-10 medium and Hank's balanced salt solution (HBSS) were purchased from Gibco BRL (Catlog No. 81200-040). 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO–AMVN), N,N′-diphenyl-p-phenylene-diamine (DPPD), 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) were obtained from Wako pure, Japan. TSII-A was a gift from new drug developing and researching center of Guangzhou traditional Chinese medical university, China.

$ONOO^-$ was synthesized by mixing aqueous solution of $NaNO_2$ and H_2O_2 in a quenched flow reactor as previously described[15] and stored at pH 11–12 till use. The concentration of $ONOO^-$ was determined with optical absorption at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$).

Isolation of LDL

LDL was isolated from fresh human plasma drawn from healthy donors under anticoagulation with 1 mg/ml of ethylenediaminetetraacetic acid (EDTA) by means of a single discontinuous density gradient ultracentrifugation procedure.[16] Then, LDL was washed at $d = 1.063$ g/ml to remove traces of contaminating albumin. Following isolation, LDL was dialyzed against 10 mM phosphate-buffered saline (PBS) (pH 7.4) containing 0.3 mM EDTA and 100 U/ml penicillin G, then filter-sterilized and stored at 4°C until use. Before the experiments, LDL was applied to a Pharmacia Sephadex G-25M PD-10 column to

remove EDTA and other low-molecular-weight contaminants, and eluted with 10 mM PBS (pH 7.4). The protein concentration of LDL solution was determined by the method of Bradford with BSA as a standard protein.[17] Then it was used immediately.

LDL Oxidation

Antioxidant properties of TSII-A were examined using four LDL oxidizing systems: (1) copper-initiated; (2) peroxyl radical-initiated; (3) $ONOO^-$-initiated; and (4) macrophage-mediated LDL oxidation systems.

In the copper-initiated oxidation system, LDL (100 μg protein/ml) was incubated with 10 mM $CuSO_4$ in the presence or absence of TSII-A in HBSS at 37°C. The LDL oxidation was evaluated by measuring TBA-reactive substances (TBARS) formation at 4 h as described elsewhere[18] and the lag time of conjugated diene formation. The lag time was determined by monitoring the absorbance at 234 nm.

In the peroxyl radical-initiated oxidation system, LDL was incubated with peroxyl radical generator, AAPH (5 mM) in the presence or absence of TSII-A in HBSS for 6 h at 37°C. The reaction was stopped by the addition of 20 μM BHT and 2 mM EDTA. LDL oxidation was measured by TBARS assay, and the change of the electrophoretic mobility of LDL was examined on 1% agarose gel using barbital buffer (pH 8.6). The gel was fixed in 5% acetic acid in 70% ethyl alcohol and stained with Sudan black B.[19] The relative electrophoretic mobility (REM) was calculated as the ratio of migration distance of treated LDL to that of control LDL.

In the $ONOO^-$-initiated oxidation system, 0.1 mg LDL in 0.5 ml of 100 mM PBS, pH 7.4 was vigorously mixed with $ONOO^-$ or with decomposed $ONOO^-$ in the presence or absence of TSII-A. Final concentration of $ONOO^-$ and decomposed $ONOO^-$ is 1.2 mM in which pH remained unchanged. After 30 min incubation at room temperature, the reaction was stopped by the addition of 20 μM BHT and 2 mM EDTA. LDL oxidation was determined by TBARS formation and by electrophoretic mobility on agarose gel.

In the macrophage-mediated oxidation system, LDL was oxidized by murine peritoneal macrophage. Resident macrophages were obtained from female C57B1/6J mice (8–12 weeks old) by peritoneal lavage with ice-cold DMEM without prior stimulation. The cells were cultured on 12-well plastic culture plates at 2×10^6 cells/well in DMEM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated (56 for 30 min) fetal calf serum (FCS), and 5% CO_2 in air at 37°C. After overnight culture, non-adherent cells were removed by washing three times with fresh DMEM medium. For the measurement of LDL oxidation, macrophages were washed three times with serum-free Ham's F-10 medium and then incubated with 100 μg/ml LDL in 1 ml of serum-free Ham's F-10 medium in the presence or absence of TSII-A. After 24 h of incubation, the medium was immediately centrifuged at 200*g* for 10 min to remove detached cells, then the supernatant was collected and 20 μM BHT and 2 mM EDTA (final concentration) were added to prevent any further oxidation. LDL oxidation was determined by TBARS formation and by electrophoretic mobility on agarose gel.

Copper Chelation Study

Copper chelating ability was determined by adding 100 μM $CuSO_4$ or water (control) to 100 μM TSII-A in HBSS solution. After incubation at room temperature for 10 min, the absorption spectra (200–700 nm) were recorded. The chelation of copper with TSII-A was evaluated by absorbance change and/or spectral shift after incubation.[20]

Superoxide Scavenging Study

The superoxide scavenging activity of the TSII-A was determined by monitoring its competition

with cytochrome *c* for superoxide using xanthine/xanthine oxidase system as superoxide generator.[21] Cytochrome *c* (10 μM), catalase (0.25 mg/ml) and xanthine (100 μM) were incubated in phosphate buffer (50 mM; pH 7.4) for 1 min, and 0.02 U/ml xanthine oxidase was added to start the reaction. Reduction of cytochrome *c* was measured spectrophotometrically at 550 nm for 3 min at room temperature. The superoxide-induced cytochrome *c* reduction rate was determined in the presence or absence of TSII-A. SOD (50 μg/ml) was used as a positive control.

Peroxyl Radical Scavenging Study

The peroxyl radical scavenging capacity of TSII-A was analyzed by DPPD assay and compared with that of Trolox. DPPD is known to react with two molecules of peroxyl radicals to give N,N′-diphenyl-p-benzoquinone diimine (DPBQ). The formation of DPBQ can be easily followed by measuring its strong absorption at 440 nm. The addition of antioxidant suppresses the formation of DPBQ by scavenging radicals, and from the competition between the antioxidant and DPPD, their relative reactivities toward the radical can be obtained.[22] DPPD (31.6 μM final concentration) was mixed with TSII-A or Trolox in acetonitrile solution and pre-warmed to 37°C. MeO–AWVN (2 mM final concentration) was added to the mixture to start the reaction and DPPD oxidation was traced at 440 nm by spectrophotometer under air at 37°C.

$ONOO^-$ Scavenging Study

$ONOO^-$ scavenging activity was assayed using the method originally described by Kooy *et al.*[23] with slight modification. TSII-A were first mixed with 100 μM diethylenetriaminepentaacetic acid and 20 μM of DHR in 50 mM of sodium phosphate buffer (pH 7.4). The solution (990 μl) was vigorously mixed with 10 μl of 2.4 mM $ONOO^-$ in 0.1 M NaOH at 25°C. Rhodamine, the resulting oxidized product of DHR, was spectrophotometrically measured at 500 nm ($\varepsilon = 78{,}780\,M^{-1}cm^{-1}$).[24] Cysteine, a peroxynitrite scavenger, was used as a positive control.[23]

Statistical Analyses

Statistical analyses were performed by using Student's *t*-test. Results are given as mean ± SD and values of $P < 0.05$ were considered significant.

RESULTS

Copper-Initiated LDL Oxidation

As shown in Table I and Figure 2, TSII-A significantly influenced copper-initiated oxidation. Lag time of the conjugated diene formation was prolonged in a dose-dependent fashion, resulting in about 1.4-, 2.0- and 5.6-fold longer than that of control at 1.0, 10 and 100 μM concentrations, respectively (Table I). TBARS formation at 4 h was 88.2 nmol TBARS/mg LDL protein in the absence of TSII-A, but decreased significantly in a concentration-dependent manner with TSII-A (Figure 2). Incubation of LDL with TSII-A alone up to 100 μM did not cause any TBARS formation (data not shown).

TABLE I Effect of TSII-A on the kinetics of copper-mediated LDL oxidation

Groups	Lag time (min)
Control	42.5 ± 6.6
TSII-A 0.1 μM	42.3 ± 15.0
1.0 μM	59.3 ± 2.1**
10 μM	85.3 ± 7.4**
100 μM	> 240**

The lag time of the conjugated diene formation was measured. LDL (100 μg/ml) was incubated with 10 μM $CuSO_4$ in the presence or absence of increasing concentrations of TSII-A in HBSS solution at 37°C for 4 h. The LDL oxidation was continuously measured by conjugated diene formation (absorbance at 234 nm). Data represent mean ± SD ($n = 3$). **$P < 0.01$ compared with control.

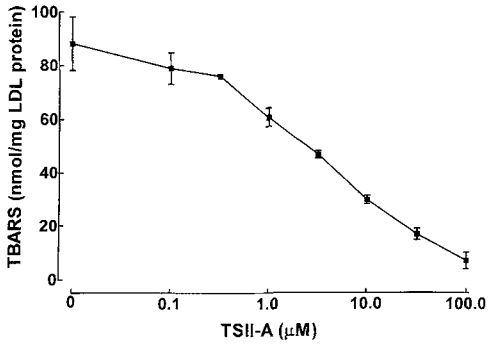

FIGURE 2 The effects of TSII-A on copper-induced LDL oxidation. LDL was oxidized by copper following the same procedures as in Table I. Oxidative modification of LDL was assayed by TBARS. Data represent mean ± SD ($n = 4$). $P < 0.01$ compared with control at 1.0 μM or higher concentrations of TSII-A.

TABLE II Effect of TSII-A on the AAPH-initiated LDL oxidation

Groups	nmol TBARS/mg LDL protein (% control)	REM
Control	45.7 ± 2.8	2.73 ± 0.46
TSII-A 0.316 μM	45.3 ± 0.6 (99.1%)	2.33 ± 0.29
1.0 μM	38.0 ± 1.3 (83.2%)**	1.53 ± 0.06*
3.16 μM	35.6 ± 0.5 (77.9%)**	1.32 ± 0.16**
10 μM	31.6 ± 1.3 (69.1%)**	1.25 ± 0.13**
31.6 μM	16.0 ± 4.3 (35.1%)**	2.37 ± 0.34
100 μM	5.2 ± 3.2 (11.4%)**	2.24 ± 0.43

LDL (100 μg/ml) was incubated with 5 mM AAPH in the presence or absence of increasing concentrations of TSII-A in HBSS solution at 37°C for 6 h. LDL oxidation LDL was assayed by TBARS and by agarose gel electrophoresis. Data represent mean ± SD ($n = 4$). *$P < 0.05$ and **$P < 0.01$ compared with control.

AAPH-Initiated LDL Oxidation

As indicated in Table II, when AAPH was used as a pro-oxidant instead of copper in the experiment, TSII-A exhibited similar ability to inhibit TBARS formation. In accordance with the TBARS data, TSII-A was able to inhibit the increased electrophoretic mobility (REM) of LDL caused by AAPH treatment in a dose-dependent manner up to 10 μM. However, the inhibition of REM by TSII-A could not be observed at higher levels (31.6 μM: 2.37 ± 0.34 and 100 μM: 2.24 ± 0.43; all $P > 0.05$ compared to control), even though TSII-A apparently inhibited TBARS formation at these concentrations. Thus we performed additional experiments to examine whether TSII-A has direct effect on the electrophoretic mobility of LDL. It was shown that electrophoretic mobility of LDL was shifted toward more negative charge with the higher concentrations of TSII-A (Figure 3).

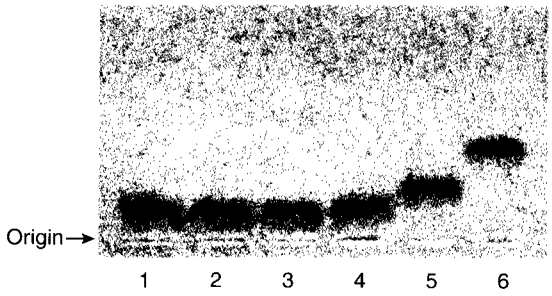

FIGURE 3 Agarose gel electrophoretic mobility of LDL incubated with TSII-A in the presence or absence of BHT. LDL was incubated with TSII-A in HBSS solution at 37°C for 30 min. After incubation, LDL samples were subjected to agarose gel electrophoresis as described in the Materials and Methods. The samples on the gel lanes are as following: lanes 1–6, LDL incubated with 0, 1.0, 3.16, 10, 31.6 and 100 μM TSII-A, respectively.

$ONOO^-$-Initiated LDL Oxidation

$ONOO^-$ increased TBARS formation and REM of LDL. Decomposed $ONOO^-$ did not show these changes. Although protective effect against ONOO-induced LDL oxidation was not evident at 10 μM or lower concentrations, TSII-A significantly suppressed TBARS formation at 31.6 and 100 μM TSII-A (Table III). Since TSII-A itself was shown to increase REM (Figure 3), we did not measure the change of REM at 31.6 and 100 μM TSII-A.

Macrophage-Mediated LDL Oxidation

As shown in Figure 4, TBARS formation of LDL was increased from 2.1 to 29.8 nmol TBARS/mg protein after 24 h incubation with macrophages. TSII-A inhibited macrophage-induced TBARS formation at concentration ranges from 0.1 to 100 μM. REM of the modified LDL showed a

TABLE III Effect of TSII-A on the $ONOO^-$-initiated LDL oxidation

Groups	TBARS (nmol/mg LDL protein)	REM
Native LDL	2.1 ± 0.5	
Decomposed $ONOO^-$	3.0 ± 1.9	nd
$ONOO^-$	9.0 ± 1.2	1.93 ± 0.10
$ONOO^-$ + TSII-A 1.0 μM	8.7 ± 0.9	1.97 ± 0.02
3.16 μM	9.4 ± 0.4	1.95 ± 0.08
10 μM	9.25 ± 1.1	2.10 ± 0.05
31.6 μM	6.2 ± 0.9**	nd
100 μM	4.4 ± 0.7**	nd

LDL (100 μg/ml) was incubated with 1.2 mM $ONOO^-$ or decomposed $ONOO^-$ in the presence or absence of increasing concentrations of TSII-A in 100 μM PBS solution at room temperature for 30 min. LDL oxidation was assayed by TBARS formation and REM. Data were expressed as mean ± SD ($n = 4$). **$P < 0.01$ compared with $ONOO^-$; nd: not determined.

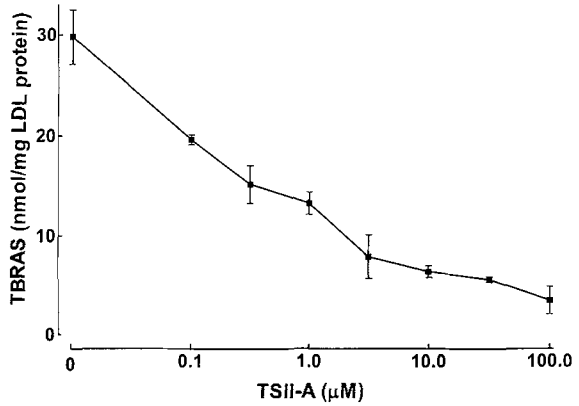

FIGURE 4 The effects of TSII-A on macrophage-induced LDL oxidation. LDL (100 μg/ml) was incubated with macrophages in the presence or absence of increasing concentrations of TSII-A in serum-free Ham's F-10 medium at 37°C for 24 h. TBARS was assayed following the same procedures as in Figure 2. Data represent mean ± SD ($n = 4$). $P < 0.01$ compared with control at 0.1 μM or higher concentrations of TSII-A.

similar pattern of inhibition to that seen in TBARS in response to TSII-A at concentration ranges from 0.1 to 10 μM (data not shown).

Peroxyl Radical Scavenging Activity

As shown in Figure 5, the absorption at 440 nm increased linearly with time due to the oxidation of DPPD by MeO–AMVN-generated peroxyl

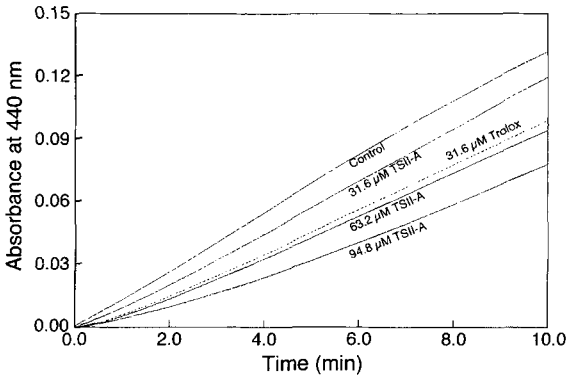

FIGURE 5 Peroxyl radical scavenging capacity of TSII-A. DPPD (31.6 μM final concentration) was mixed with or without TSII-A in acetonitrile solution and pre-warmed to 37°C. MeO-AWVN (2 mM final concentration) was finally added to the mixture to start the reaction under air at 37°C. DPPD oxidation was traced at 440 nm by spectrophotometer.

radical. The oxidation rate of DPPD was 0.0143 ± 0.0008 OD/min. TSII-A dose-dependently suppressed DPPD oxidation and the suppression was about 1/2 of that by Trolox at the same concentration (31.6 μM). Indeed, the oxidation rate was 0.0128 ± 0.0003 ($P < 0.05$, $n = 4$), 0.0105 ± 0.0002 ($P < 0.01$, $n = 4$), 0.0083 ± 0.0004 ($P < 0.01$, $n = 4$) and 0.0113 ± 0.0006 OD/min ($P < 0.01$, $n = 4$) in the presence of 31.6, 63.2, 94.8 μM TSII-A and 31.6 μM Trolox, respectively.

Copper Chelation Study

TSII-A itself has peak absorption at 280 nm. Addition of $CuSO_4$ did not cause any spectral shift or absorbance change of TSII-A compared to control (data not shown), indicating TSII-A does not chelate copper.

Superoxide Scavenging Study

Superoxide generated by xanthine oxidase caused cytochrome *c* reduction at a rate of 0.0903 ± 0.0005 OD/min. This reduction was completely inhibited by SOD (50 μg/ml). TSII-A did not inhibit cytochrome *c* reduction at concentrations of 1.0, 10 and 31.6 μM. It rather slightly but

not significantly increased cytochrome *c* reduction rate. Indeed, the cytochrome *c* reduction rate was 0.0906 ± 0.0003, 0.0975 ± 0.0025 and 0.1000 ± 0.0026 OD/min in the presence of 1.0, 10 and 31.6 μM TSII-A, respectively (all $P > 0.05$ compared with control, $n = 4$). Incubation of cytochrome *c* alone with TSII-A caused a small increase in the absorbance at 550 nm, which was not influenced by SOD (50 mg/ml) plus catalase (50 mg/ml) (data not shown).

$ONOO^-$ Scavenging Study

In the presence of $ONOO^-$, DHR was oxidized to rhodamine which increased absorption at 500 nm dose-dependently. Cysteine effectively inhibited $ONOO^-$-induced oxidation of DHR with IC_{50} of 19 μM. However, TSII-A did not exert any $ONOO^-$ scavenging activity even at 100 μM (data not shown).

DISCUSSION

This study demonstrates that TSII-A can effectively protect LDL against oxidation induced by four different oxidative challenges with different characteristics. As measured by TBARS formation, TSII-A inhibited copper-, AAPH-, $ONOO^-$-, and macrophage-induced LDL oxidation by 80.8%, 65.0%, 31.1%, and 81.2% respectively at 31.6 μM. Regarding the mechanisms underlying its antioxidant effect, our results indicate that the inhibition of LDL oxidation by TSII-A is related neither to metal ion chelation[12] nor to direct superoxide scavenging activity, which is in agreement with the previous report.[11] The inhibitory effect of TSII-A on $ONOO^-$-induced LDL oxidation is also not directly related to scavenging activity toward $ONOO^-$ itself by the drug.

It is known that the generation of peroxyl radical is a necessary step in the formation of TBARS during lipid peroxidation.[25] Our finding that TSII-A inhibited AAPH-induced LDL oxidation suggest that TSII-A possesses peroxyl radical scavenging activity. As was expected TSII-A suppressed MeO–AMVN-induced DPPD oxidation (Figure 5), working as a peroxyl radical scavenger. However, the degree of inhibition in DPPD oxidation is not fully concordant with that of the inhibition in LDL oxidation, suggesting an involvement of other mechanisms besides its peroxyl radical scavenging activity. These may include lipid radical scavenging property, since TSII-A has been shown to scavenge lipid radicals in the lipid peroxidation process of myocardial mitochondrial membranes.[11] Furthermore, TSII-A-induced conformational change of LDL particles through binding to LDL appears to be an additional mechanism based on our finding that TSII-A increased negative charge of LDL particles (Figure 3). In view of the lipophilic nature of TSII-A, such binding of TSII-A to LDL may alter the availability of lipid to oxidation as was shown in fluvastatin-induced inhibition of LDL oxidation.[26] It was recently reported that lipophilic drug, fluvastatin can bind to LDL and alter the charge of LDL particles, which results in the change in lipoprotein physicochemical characteristics and contributes to the antioxidative effect of this drug against LDL oxidation.[26]

The above mentioned effect of TSII-A on the charge of LDL particles also explains the confusing results in Table II. Namely the reason for the non-parallel effects of high concentration of TSII-A on TBARS formation and REM of LDL was due to its direct effect on LDL. TSII-A itself modifies LDL structure toward more negative charge and increases REM at high concentrations, which counteracts the decrease of REM caused by inhibition of LDL oxidation.

In conclusion, this is the first paper showing TSII-A is an effective inhibitor of LDL oxidation *in vitro*, as demonstrated by its inhibitory ability in four different oxidative challenge-induced LDL oxidation.

Acknowledgments

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