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Prooxidant activity of caffeic acid, dietary non-flavonoid phenolic acid, on Cu^{2+}-induced low density lipoprotein oxidation

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Abstract The effects of caffeic acid (CA) and chlorogenic acid (CHA), an ester of caffeic acid with quinic acid, were studied on isolated human Cu^{2+}-induced low density lipoprotein (LDL) oxidation in initiation and propagation phases by measuring the formation of thiobarbituric acid reactive substances (TBARS), detecting conjugated diene and investigating the electrophoretic mobility change of LDL. Both non-flavonoids exhibited prooxidant and antioxidant activities depending on the LDL oxidation phases. CA and CHA (0.1 μM or more) enhanced LDL oxidation in the propagation phase. In agreement with previous findings, 0.5 μM CA and CHA inhibited LDL oxidation in the initiation phase. When 0.5 μM CA was added at 0 min, the duration of inhibition was about 60 min. Yet, after >9 min incubation with Cu^{2+}, 0.5 μM CA accelerated LDL oxidation. The acceleration ratios were modified depending on the oxidation process and the concentration of added CA in the propagation phase. The maximum acceleration ratio was about 5 on addition of 2–5 μM CA, attained after 40 min incubation with Cu^{2+}. Even in the propagation phase, an elevated concentration of CA inhibited oxidation; after 20 min incubation with Cu^{2+}, CA at >3 μM functioned as an inhibitor. Further studies must be performed in order to clarify the counteracting deleterious prooxidant conditions of these widespread natural dietary compounds.

Key words: Caffeic acid; Chlorogenic acid; Ascorbic acid; Prooxidant; Antioxidant; Low-density lipoprotein

1. Introduction

Caffeic acid (CA) and chlorogenic acid (CHA) are non-flavonoid phenolic compounds from plants and are found naturally in various foodstuffs and beverages such as coffee beans and their soluble constituents, potatoes, fruits like apples and their juices, tobacco leaves, olive oil and wines [1–4]. There is growing interest in the multiple biological and pharmacological properties of non-flavonoid phenolic compounds such as CA and CHA [5]. They have been reported to have anti-inflammatory, cocarcinogenic, antimutagenic and anticarcinogenic activities [4,6–9]. Particular attention has been paid to the antioxidative actions of these widespread dietary phenolic compounds [4–12]. CA and CHA have also been reported to stimulate hydroxyl radical formation and reduce ferrylmyoglobin [13–15], which suggests their potential prooxidant action.

In the pathogenesis of atherosclerosis, recent reports suggest that oxidative modification of low-density lipoprotein (LDL) may play an important role in the initiation and progression of atherosclerosis and that lipid peroxidation has a key role in its development [16]. Oxidation of LDL leads to its uptake by a scavenger receptor on the surface of macrophages that subsequently might result in foam cell formation [16]. Endothelial cells and macrophages are known to accelerate LDL oxidation [17–19]. LDL oxidation is also catalyzed by heavy metals such as Cu^{2+} and Fe^{3+} [20,21]. ln contrast, antioxidants such as probucol and ascorbic acid were shown to prevent LDL oxidation [22,23] and probucol was found to protect against spontaneous atherosclerotic changes [24]. The above-described observations provide evidence that oxidation of LDL is involved in the initiation and progression of atherosclerosis and that protection against LDL oxidation is critical to prevent the development of atherosclerosis.

Autoxidation of LDL is a free radical chain reaction which is known to consist of the phases of initiation, propagation and termination reactions [25]. Recently, we established that catechins such as (−)-epicatechin and (−)-epigallocatechin (flavan-3-ol derivatives, flavonoids abundant in green tea leaves) possess the antioxidative and prooxidative characteristics of Cu^{2+}-induced LDL oxidation during the process of oxidation [26]. In the initiation phase, LDL oxidation was inhibited by catechin addition. ln contrast, during the propagation phase of LDL oxidation, catechins worked as accelerators of oxidation. Therefore, in the present study, we investigated the effects of CA and CHA on the initiation and propagation phases. We found that during the initial phase of LDL oxidation the duration of inhibition was gradually reduced with respect to the time of addition of CA or CHA and finally, that CA and CHA exerted accelerative effects on the propagation phase. Nevertheless, the concentration of non-flavonoids modified the prooxidative and antioxidative activities.

2. Materials and methods

2.1. Chemicals

CA (3,4-dihydroxycinnamic acid) and CHA (3-(3,4-dihydroxycinnamoyl)quinic acid) were obtained from Sigma (St. Louis, MO). 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5), which generates nitric oxide (NO), was from Dojin Chemicals (Kumamoto, Japan). Ascorbic acid (3-oxo-L-gulofuranolactone) and other chemicals were from Wako Pure Chemicals (Tokyo, Japan).

2.2. Isolation of human LDL

LDL was prepared from human plasma of healthy donors by ultracentrifugation as described [27] with a density of cut-off 1.005–1.065 and then dialyzed with 20 mM sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride and 2 μM EDTA to prevent the oxidation of LDL [28]. LDL was stocked in N_2 gas saturated vials at 4°C and used within 1 week. Healthy donors gave their informed consent according to the Declaration of Helsinki. Protein concentration of LDL was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as standard.

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2.3. LDL oxidation

LDL solution (0.2 mg protein/ml) was prepared by dilution of isolated LDL solution with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride and 0.2 μM EDTA (PBS-EDTA). LDL solutions were incubated with 5 μM $CuSO_4$ at 37°C in the presence or absence of CA or CHA in the initiation and propagation phases of oxidation.

2.4. Measurement of LDL peroxidation

Lipid peroxidation was estimated as thiobarbituric acid-reactive substances (TBARS) fluorometrically and/or measured by absorbance at 234 nm to detect conjugated dienes [28]. In TBARS analysis, malondialdehyde (MDA) formed from 1,1,3,3-tetraethoxypropane was used as a reference standard and the results were expressed as nmol equivalent of MDA. These experiments were repeated two or three times, the results obtained being almost the same. Sample-to-sample variations from different volunteers were recognized. Typical results are shown in the figures.

2.5. Agarose gel electrophoretic mobility change of LDL

Agarose gel electrophoresis was performed to evaluate the electrophoretic mobility of LDL. Aliquots (50 μg/250 μl of LDL protein) were concentrated 5-fold using an Ultrafree Concentrator (C3LCC, Millipore). This was performed by centrifugation at 5000×*g* for 120 min at 4°C. 8 μl of the concentrated samples were applied to 1% agarose gel electrophoresis. The gel was stained with 0.25% Coomassie Brilliant blue in 7% acetic acid.

3. Results

The effects of 0.5 μM CA and CHA were examined on Cu^{2+}-induced LDL oxidation during the initiation (0 min) and propagation (20 min incubation with Cu^{2+}) phases. As shown in Fig. 1A,B, CA and CHA inhibited LDL oxidation in the initiation phase consistent with previous reports [5,11,12]. In contrast, it is evident that in the propagation phase, both CA and CHA accelerated LDL oxidation. However, 5 μM CA inhibited LDL oxidation even in the propagation phase (Fig. 1A) and CHA also exhibited the same effect (data not shown). 0.5 μM ascorbic acid (non-phenolic acid) also displayed the same antioxidative and prooxidative effects on LDL oxidation as elicited by CA and CHA (Fig. 1B). 5 μM ascorbic acid inhibited oxidation during the propagation phase (20 min after Cu^{2+} addition) (data not shown). Nevertheless, NO released from NOC5 inhibited even CA-accelerated LDL oxidation (Fig. 1A), which was also observed in the case of catechins (flavan-3-ol derivatives, flavonoid) [26]. NO functioned as an antioxidant during all phases of LDL oxidation, even when present at low concentration (data not shown). 0.5 μM ascorbic acid did not inhibit CA- and CHA-accelerated LDL oxidation, but rather accelerated the effects of CA and CHA (data not shown). As shown in Fig. 2, the effect of CA concentration was examined on changes in electrophoretic mobility of LDL on agarose gel electrophoresis for LDL oxidation. The mobility changes were accelerated by CA (0.5 and 1.0 μM) in the propagation phase (Fig. 2). However, 5.0 μM CA inhibited the mobility change. These results indicated that higher concentrations of CA inhibited the oxidation and mobility even in the propagation phase, in accordance with the TBARS formation shown in Figs. 1A and 4. CHA also demonstrated the same tendency, but CA was more effective (data not shown). The effects of CA on LDL oxidation were examined with respect to time after Cu^{2+} addition by following the formation of TBARS and detection of conjugated dienes (Fig. 3A,B). 0.5 μM CA additions at 0–6 min incubation with Cu^{2+} inhibited the formation of both TBARS and conjugated dienes. The duration of inhibition gradually decreased. After 12 min incubation of LDL with Cu^{2+}, CA accelerated the oxidation, the rate of acceleration gradually increasing up to 20 min incubation with Cu^{2+} (Fig. 3). As shown in Fig. 4, the maximum rate of acceleration and ratio were obtained at 40 min

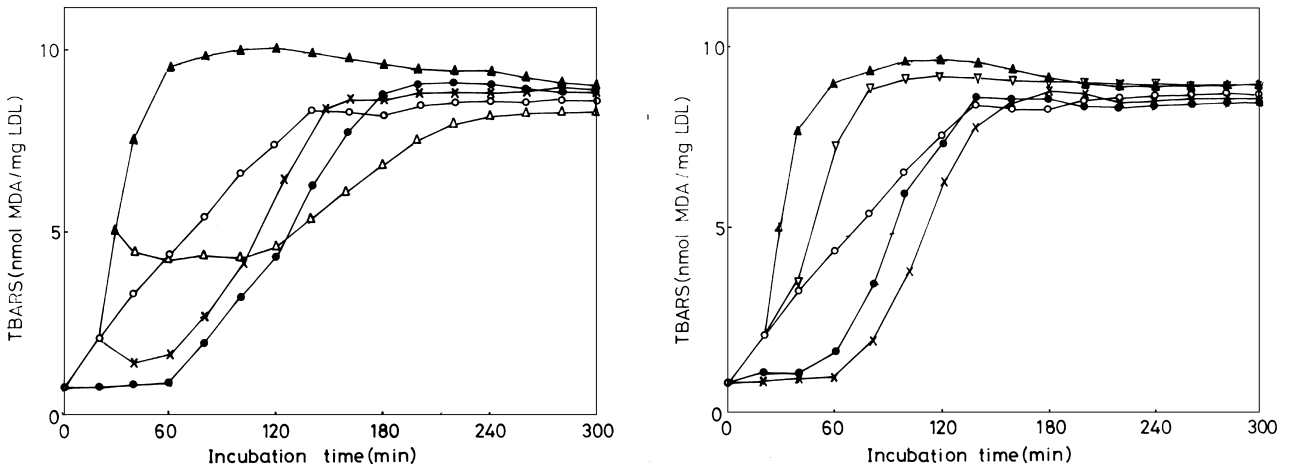

Fig. 1. Accelerative and inhibitory effects of CA and CHA on Cu^{2+}-induced TBARS formation in LDL. Left: Effects of 0.5 and 5.0 μM CA in the propagation phase and inhibitory effect of NO released from NOC5 on CA accelerated Cu^{2+}-induced LDL oxidation. LDL (0.2 mg protein/ml) was dissolved in 10 ml of PBS-EDTA and the reaction was initiated by adding 5 μM $CuSO_4$ at 37°C. The reaction mixture (R.M.) (10 ml) was used to measure TBARS formation as control (○). After 20 min incubation, R.M. (4.0 ml) was separated and 0.5 μM CA was added (▲). 10 min after the addition of 0.5 μM CA, 2.0 ml R.M. with 0.5 μM CA was removed and 5 μM NOC5 was added (△). After 20 min incubation of R.M. (2.0 ml), 5.0 μM CA was added (×). In 2.0 ml of R.M., 0.5 μM CA was added at 0 min as the initiation phase (●). TBARS formation was measured at 20-min intervals using an aliquot (100 μl) as described in Section 2. Right: Effects of 0.5 μM CHA and ascorbic acid in initiation and propagation phases. LDL (0.2 mg protein/ml) was dissolved in 10 ml of PBS-EDTA and the reaction was initiated by adding 5 μM $CuSO_4$ at 37°C, designated R.M. TBARS formation was measured at every 20 min using an aliquot (100 μl) as described in Section 2. R.M. (6.0 ml) was used to measure TBARS formation as control (○). After 20 min incubation, R.M. (2.0 ml portions) was removed and 0.5 μM CHA (▲) or 0.5 μM ascorbic acid was added (▽). To each 2.0 ml aliquot of R.M., 0.5 μM CHA (●) or 0.5 μM ascorbic acid (×) was added at 0 min as the initiation phase.

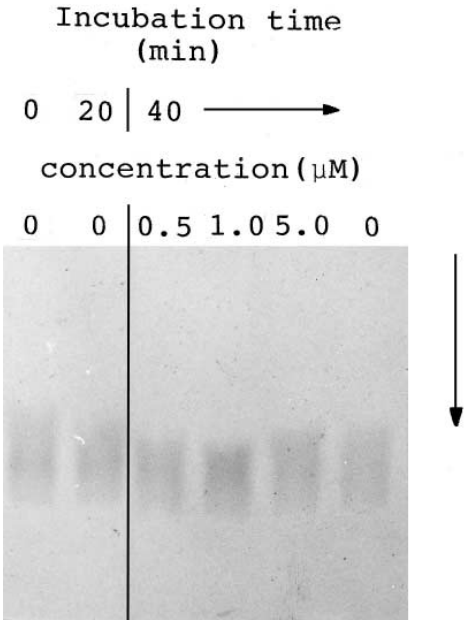

Fig. 2. Cu^{2+}-induced mobility changes of LDL using 1% agarose gel electrophoresis in the presence of various concentration of CA. 1 ml of each LDL (0.2 mg protein/ml in PBS-EDTA) solution was incubated with 5 μM $CuSO_4$ at 37°C. After 20 min incubation, the indicated concentration of CA was added to each reaction mixture. After 20 min incubation with or without the indicated concentration of CA, 250 μl of the reaction mixture was removed to perform agarose gel electrophoresis as described in Section 2. 250 μl of the 0 and 20 min incubation samples without CA addition were also used as control. 0.1 mM EDTA was added to stop the reaction.

incubation. The acceleration and inhibition ratios in the propagation phase of LDL oxidation as measured by TBARS formation varied as a function of CA concentration and time of oxidation (Fig. 4). After 20 min incubation of LDL with 5 μM Cu^{2+} (●), 0.1 μM CA accelerated the oxidation process, the ratio being about 1.7 and the maximum acceleration ratio amounted to approx. 2.9 at 0.5–2.0 μM CA. However, CA at >3.0 μM tended to inhibit oxidation, even in the propagation phase. 5.0 and 10 μM CA inhibited LDL oxidation and the acceleration ratio was near to -2.0, indicating that CA reduced the extent of TBARS formation as shown in Fig. 1A. The decrease in TBARS formation due to CA might be explained as follows. Phenolic compounds such as CA withdraw peroxyl and/or alkoxyl radicals from the chain reaction [9] and the hydroperoxide derivatives formed might be reversed during the initial phase. The maximum acceleration ratio with CA was about 5, which was attained at 40 min incubation of LDL with Cu^{2+} during the propagation phase and the CA concentration was in the range 2.0–5.0 μM. Incubation of LDL for 60–100 min with Cu^{2+} in the propagation phase gradually lowered the acceleration ratio. Nevertheless, CA even at 10 μM still resulted in acceleration, the ratio being about 1.5–3.5 (△, ▲ and ×).

4. Discussion

In accordance with previous papers [5,9–12], the non-flavonoid, naturally abundant dietary phenolic compounds, CA and CHA, were confirmed to inhibit Cu^{2+}-induced LDL oxidation in the initiation phase. However, during the oxidation step from initiation to propagation, we found the duration of inhibition by CA to decrease gradually, the inhibitory effect being completely abolished (Fig. 3). Furthermore, we observed that CA and CHA accelerated the rates of LDL oxidation in the propagation phase. This finding means that CA and CHA exert prooxidant activities against free radical chain reactions such as lipid peroxidation. Laranjinha et al. [5], even

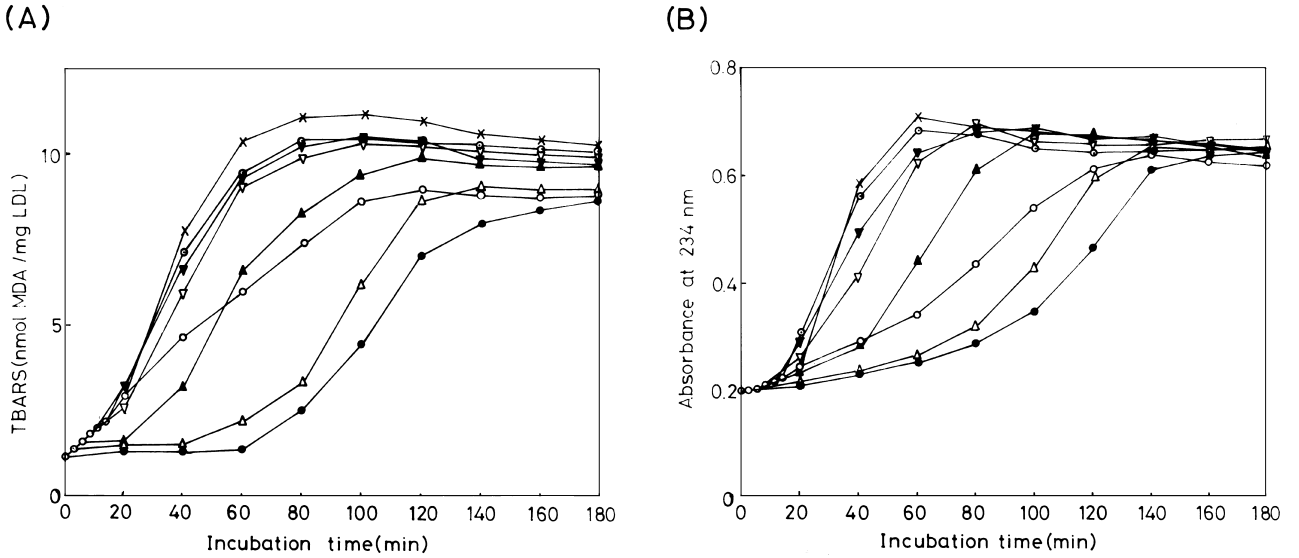

Fig. 3. Inhibitory and accelerative modifications of CA effects on Cu^{2+}-induced LDL oxidation in the initiation and propagation phases. (A) Effects on TBARS formation. LDL (0.2 mg protein/ml) was dissolved in 20 ml PBS-EDTA and the reaction was initiated with the addition of 5 μM $CuSO_4$ at 37°C. A 2.0 ml aliquot was removed and 0.5 μM CA was added every 3 min except for the 20 min incubation sample. A 100 μl aliquot was taken from each reaction mixture at 20-min intervals after Cu^{2+} addition in order to measure TBARS formation as described in Section 2. (○) No CA, (●) 0.5 μM CA at 0 min, (△) 0.5 μM CA at 3 min, (▲) 0.5 μM CA at 6 min, (▽) 0.5 μM CA at 9 min, (▼) 0.5 μM CA at 12 min, (⊙) O.5 μM CA at 15 min, (×) 0.5 μM CA at 20 min. (B) Effects on conjugated diene formation. LDL (0.2 mg protein/ml) was dissolved in 32 ml PBS-EDTA and the reaction was initiated as described in A. A 4.0 ml aliquot was removed and 0.5 μM CA was added as described in A. Formation of conjugated diene was measured at 20-min intervals as described in Section 2. All symbols correspond to those in A and under the same experimental conditions.

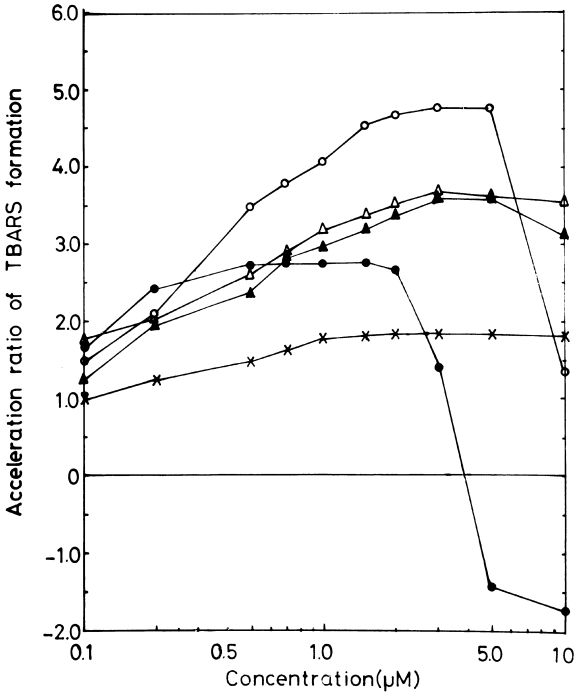

Fig. 4. Changes of acceleration and inhibition ratios as a function of CA concentration added and time course of the propagation phase in LDL oxidation. LDL (0.2 mg protein/ml) was dissolved in 24 ml PBS-EDTA and the reaction was initiated by the addition of 5 μM $CuSO_4$ at 37°C. At 20-min intervals (20, 40, 60, 80 and 100 min incubation), an aliquot (0.4 ml) was removed and 0–10 μM CA was added, followed by incubation for another 20 min. An aliquot (100 μl) from each reaction mixture was then taken in order to measure TBARS formation as described in Section 2. The acceleration or inhibition ratio at the indicated time of CA addition was calculated from the corresponding TBARS formation rate for 20 min incubation with CA against 0. (●) 20 min, (○), 40 min, (△) 60 min, (▲) 80 min, (×) 100 min.

though they did not provide data, reported that non-flavonoids such as CA or CHA were probably added during the propagation step of LDL peroxidation. However, they did not observe an accelerative effect, but rather an inhibitive effect because of the higher concentration of non-flavonoid. The prooxidant characteristics of CA and CHA were suggested in previous papers [13–15]. The effects of CA and CHA on the Fenton reaction were examined by using the ESR spin trapping technique [13]. CA and CHA (10 μM to 10 mM level) stimulated the formation of hydroxyl radicals in a reaction mixture containing H_2O_2, $FeCl_3$ and EDTA. However, in the presence of 3-hydroxyanthranic acid with H_2O_2 and $FeCl_3$, CA and CHA resulted in a decrease of hydroxyl radical formation [14]. CA and CHA reduced ferrylmyoglobin and, during the electron-transfer reactions, CA and CHA were oxidized to quinoid forms [15]. The above reports indicate that CA and CHA exert apparently conflicting effects depending on the oxidation conditions and that they are potential prooxidants.

In our experiments, 0.75 μM CA and 0.2 μM EDTA without Cu^{2+}, or 0.75 μM CA, 0.2 μM EDTA and 1 mM H_2O_2 without Cu^{2+} did not stimulate LDL oxidation (data not shown). The results indicate that the Fenton reaction might not be involved and that Cu^{2+} is essential in our system. In the propagation phase at 20 and 40 min incubation with Cu^{2+}, the accelerative effects of 0.75 μM CA clearly remained unchanged on the addition of 1 mM H_2O_2; the addition of catalase (1–10 units/ml) to the CA-accelerated LDL oxidation system was also without effect (data not shown). Therefore, in our experimental systems, iron, EDTA and H_2O_2 might not be related. Changes in Cu^{2+} concentration (0.5–5.0 μM) with 1.0 μM CA did not affect the duration of inhibition and initial rate of TBARS formation during the initiation phase (0 min CA addition) (data not shown). The results suggest that chelation of copper ion with CA might not be critical for the effects of CA as suggested by Nardini et al. [11].

Ascorbic acid is known to switch from anti- to pro-oxidant activity, depending on its concentration and on the presence of free transition metal ions [29]. Frei reported that ascorbic acid completely protected lipid in plasma and LDL against detectable peroxidative damage and was reactive enough to intercept oxidants in the aqueous phase before they could attack and cause detectable oxidative damage to lipids [30]. However, in our experiment (Fig. 1B), ascorbic acid displayed antioxidative and prooxidative activities depending on the oxidation state of LDL, similar to the effects demonstrated by CA and CHA. The 0.5 μM CA-accelerated propagation rate was further stimulated by 0.5 μM ascorbic acid (data not shown), indicating that ascorbic acid did not work as an antioxidant. Phenolic compounds are known to quench $OH^{\bullet}$ quite efficiently and are also supposed to withdraw peroxyl and alkoxyl radicals from the chain reaction that consequently breaks down the autoxidation process [9]. Moreover, even α-tocopherol, a well-known antioxidant, has been reported to stimulate Cu^{2+}-initiated lipid peroxidation when phospholipids and traces of their hydroperoxide derivatives are included [31].

In our experiments, non-flavonoids (CA and CHA) as well as catechins (flavan-3-ol derivatives, flavonoids) [26] and ascorbic acid (non-phenolic compound) have been shown to inhibit Cu^{2+}-induced LDL oxidation in the initiation phase. However, in the propagation phase, the above-described compounds worked as prooxidants and exhibited same trends. Depending upon the redox status, non-flavonoids might form reactive oxidation products such as semiquinones and quinones and function to stimulate oxidative reactions. All such common natural foodstuffs and beverages should be carefully studied in ordrer to determine the conditions of developing deleterious effects, since prooxidant states may play important roles in atherogenesis, aging and cancer.

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