Free Radical Research, March 2006; 40(3): 321–331

Fibrinogen is a co-antioxidant that supplements the vitamin E analog trolox in a model system

NTEI ABUDU[1], JAMES J MILLER[1], & STANLEY S. LEVINSON[1,2]

[1]*Department of Pathology and Laboratory Medicine, University of Louisville, Louisville, KY 40292, USA, and* [2]*Laboratory Service, VAMC, 800 Zorn Avenue, Louisville, KY 40206-1466, USA*

Accepted by Professor J. Viña

(Received 27 June 2005; in revised form 28 October 2005)

Abstract

Objective: It appears that the atherosclerotic plaque is a prooxidant environment where some molecules that are normally antioxidants, including vitamins C and E, may act as prooxidants that contribute to atherosclerosis by oxidizing LDL. Some molecules can act as co-antioxidants to eliminate this prooxidant effect by recycling or other mechanisms of supplementation. Fibrinogen and other acute phase proteins found in the plaque are antioxidants. We hypothesized that fibrinogen can act as a co-antioxidant to supplement vitamin E thereby eliminating its oxidative effect under prooxidant conditions. We tested a model system for this hypothesis using the vitamin E analogue Trolox in a cell free system.

Methods: LDL was oxidized using 5 umol/l copper. Antioxidant conditions were achieved by adding the antioxidants immediately with LDL, while prooxidant conditions were created by adding antioxidants after a 40 min delay. Oxidation was monitored as the lag phase at 234 nm.

Results: Under antioxidant conditions, the protective effect of fibrinogen and Trolox combined together were about equal to the sum of the anitioxidant effects of each alone (additive), while under prooxidant conditions the combined protection was 54–200% greater (synergistic). These effects were different than those of vitamin C with Trolox in that under antioxidant conditions fibrinogen and Trolox were additive while vitamin C and Trolox showed strong synergistic effects, and in that unlike vitamin C and Trolox fibrinogen showed no prooxidant tendencies under prooxidant reaction conditions.

Conclusions: The data indicated that fibrinogen did act as a co-antioxidant to supplement Trolox and eliminate its prooxidant effect, most probably, by directly quenching the phenoxyl radical, because unlike vitamin C, fibrinogen did not appear to recycle vitamin E. But fibrinogen may act as a universal antioxidant, since unlike Trolox and vitamin C, it showed little tendency toward becoming a prooxidant.

Keywords: *Fibrinogen, co-antioxidants, vitamin C, vitamin E, transition metals*

Introduction

Much evidence indicates that atherosclerosis is a process of arterial injury, inflammation and thrombosis [1,2], and that elevated beta-lipoproteins are an integral part of this process [1–3]. Moreover, it has been hypothesized that oxidized (Ox) LDL is a primary ingredient in the initiation and propagation of atherosclerosis [1,4].

Vitamin E in the form of alpha-tocopherol is the major lipid soluble antioxidant in LDL and vitamin C has proved to be superior to other water-soluble antioxidants at protecting LDL from free radicals and other reactive species [5,6]. Because of abundant evidence supporting the oxidation of LDL hypothesis of atherosclerosis, it was widely thought that ingested antioxidant vitamins would retard atherosclerosis in humans [5,6], but randomized secondary prevention studies largely failed to find a reduction in heart disease with vitamin therapy [5–7].

One reason that has been widely proposed to explain this failure is because many small molecular

Correspondence: S. S. Levinson, Laboratory Service, VAMC, 800 Zorn Avenue, Louisville, KY 40206-1466, USA. Tel: 1 502 287 5565/5572. Fax: 1 502 287 6265. E-mail: levinson@louisville.edu

ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500488998

weight antioxidants manifest radical forms themselves as a result of one electron reactions and, under certain conditions, can act as prooxidants [5,7]. Both vitamin E and ascorbic acid (vitamin C) can facilitate oxidation under prooxidant conditions [8–10]. Moreover, dehydroascorbate, uric acid, flavanoids, and even glutathione can promote reactive species [10–12]. Because the atherosclerotic plaque appears to be a prooxidant environment containing transition metals, seeding peroxides, and white blood cells which contain enzymes that produce reactive species [7,13,14], these antioxidant radical intermediates may accelerate rather than retard atherosclerosis within the plaque [7,15].

Experimentally, in cell free systems, a prooxidant effect has most consistently been demonstrated by delayed addition of an antioxidant to a reaction solution consisting of a transition metal oxidizing agent and lipid after the oxidation process has already begun (prooxidant condition)[10,11,16]. The addition of antioxidants at the start of the reaction usually only causes antioxidant effects (antioxidant condition).

Under normal conditions where cells are intact and healthy, and in plasma, the prooxidant tendencies of these molecules may be buffered by interactions with other antioxidants—so called supplemental or co-antioxidants [17]. These include β-carotene, ubiquinol-10, 3-hydroxyanthranilic acid, bilirubin, and vitamin C [6,7,9,17]. In some cases, a synergistic effect has been achieved where, in the presence of a co-antioxidant, the antioxidant effect of a mixture is greater then the antioxidant effect of the sum of each antioxidant alone [7,18]. Vitamin C seems to be an especially important synergistic antioxidant, since it can recycle the vitamin E radical back to the reduced form [19]. The failure to date of antioxidant cocktails to retard disease may be related to these complex interactions of pro- and antioxidant capabilities by biological molecules in the prooxidant environment of the plaque.

Both albumin and native fibrinogen are found in abundance in the plaque [20,21]. Albumin in conjunction with bilirubin (natural albumin) is a powerful antioxidant that can inhibit metal catalyzed oxidation and appears to be a supplemental antioxidant that is able to recycle vitamin E radical [22,23].

Fibrinogen has been shown to be an efficient scavenger of reactive oxygen species [24,25], and neutralizes reactive nitrogen species [26]. Fibrinopeptide B contains the amino acids arginine and phenylalanine that readily accept electrons and has been shown to exhibit anti-inflammatory properties [25]. In the absence of bilirubin, albumin retards oxidation sluggishly, but fibrinogen shows a rapid propagation phase, more similar to vitamin C and vitamin E [25]. This antioxidant property is most likely due to the unique architecture of fibrinogen allowing for the fibrinopeptides to scavenge free radicals.

Because of these features, we hypothesized that fibrinogen would not show prooxidant behavior under either antioxidant or prooxidant conditions. Moreover, we hypothesized that fibrinogen would act as a co-antioxidant that would synergistically supplement vitamin E and eliminate its tendency for oxidizing lipoproteins under prooxidant conditions. To explore these hypotheses further, in this study, we investigated the biochemical co-antioxidant interactions between the vitamin E analogue Trolox and fibrinogen for protecting LDL in a model cell free system during copper mediated oxidation under antioxidant and prooxidant conditions. Also, to obtain a better idea as to whether or not fibrinogen might have a propensity to recycle vitamin E, we compared the reaction effects between fibrinogen and Trolox with those between vitamin C, that is known to recycle Trolox, and Trolox under both antioxidant and prooxidant conditions.

Regarding the second hypothesis, we caution that the major physiological form of vitamin E is fat-soluble alpha-tocopherol that acts at the membrane surface to transfer an electron and these experiments were conducted in a cell free system using Trolox for our source of phenolic group. Thus, in these experiments, we were examining the biochemical, not the physiological, validity for these reactions. If fibrinogen did not act as a co-antioxidant in aqueous solution with free access to Trolox, it would be most unlikely that it would act as a co-antioxidant with LDL bound vitamin E and it would not appear to be worth pursuing this hypothesis further. This approach seems a reasonable first step in investigating these questions.

Materials and methods

Samples and lipoprotein isolation

The study was approved by the University of Louisville and VAMC committees for protection of human rights for performance with left over, unused fresh human blood from the clinical laboratory.

Serum was obtained by centrifugation at room temperature. Sera collected in red top tubes (without anticoagulant), from patients being tested for routine lipid profiles, were pooled into lavender tubes containing potassium EDTA and kept refrigerated for up to 5 days. Tubes containing triglyceride concentrations >1500 mg/l were excluded. LDL ($d = 1.020–1.063$) was isolated by the standard sequential ultracentrifugation flotation procedure at 100,000g using sodium bromide [27], for 18 h at $d = 1.020$ to remove IDL and VLDL and again for 18 h at 1.063 to float LDL. The LDL preparation was dialyzed against 0.01 mol/l phosphate buffered saline (PBS, Sigma, St Louis, MO), containing 100 umol/l EDTA, and reconstitution of all volumes during LDL

isolation was made using the same EDTA solution. Some of the LDL solution was used to measure apo B [24] and absorbance at 280 nm, and to test for purity. The rest was stored frozen at −70°C in aliquots for these experiments for up to 2 months. Agarose lipoprotein and protein electrophoresis of the LDL solution at a concentration of apo B near that used in the assay and at a 10X concentrated level of apo B showed a high degree of purified LDL for each preparation migrating mainly as a LDL band in the beta-region of the gels with no apparent contamination by albumin.

Measurement of oxidation

The lag phase was the measure of oxidation used to compare antioxidant effects. Assessment of LDL oxidation by continuously monitoring conjugated diene formation at 234 nm is a highly reproducible, widely used approach that has been shown by many studies to correlate quite well with other methods of measurement of lipoprotein oxidation [7,25,28]. In a previous report, we showed that measurement of lag phase by following the absorbance at 234 nm was an accurate reflection of inhibition by antioxidants in our copper catalyzed cell free system as indicated by good agreement with two other standard biochemical measurements of oxidation—changes in peroxide formation (xylenol orange) and electrophoretic mobility [25]. Therefore, in the present study, we measured only conjugated diene formation. All experiments were run in duplicate and repeated in duplicate for a total of 4 replicates. The results within an experiment were very similar, and, although between repeat experiments there was greater variability, the final effects were similar. Moreover, the conclusions from repeat experiments were always the same.

Antioxidant solutions, concentrations of reactants and assay conditions

Immediately, prior to each experiment, EDTA was removed by gel filtration (Micro Biospin 30 columns, Biorad, Hercules, CA) using PBS, and antioxidant solutions were freshly prepared and kept cold. The antioxidants used were immunoelectrophoretically pure fibrinogen (Sigma), the vitamin E analogue Trolox (Aldrich of Sigma-Aldrich Chemical Company), and vitamin C (Sigma) [25].

The final concentration of apo B used in the oxidation reaction was nearly 30 mg/l [25] (For each experiment, the necessary dilution was calculated from the absorbance relationship at 280 nm between absorbance and the apo B concentration). We used a spectrophotometer containing 8 cells (Spectronic, Milton Roy, Rochester, NY). The reaction was started in each reaction cuvet by adding 10 uL of $CuSO_4$ to a final concentration of 5 umol/l to a 1 mL solution of LDL. Also 10 uL of each anitoxidant was added to 1 mL of LDL solution in the cuvet. When the antioxidants were added at zero time along with the copper, all antioxidants elongated the lag phase of LDL beyond that of copper alone. We call this the antioxidant condition. When the antioxidants were added with a 40 min delay after the LDL and copper, propagation was initiated in the cuvet containing Trolox before the cuvet containing only copper and LDL. We call this the prooxidant condition. All experiments contained duplicates and were repeated twice.

In these experiments, we used 30 mg/l of apo B in lipoprotein and 50 mg/l of fibrinogen in the reaction solution. The relative concentrations of the two are similar to that found in plasma but the absolute concentrations are diluted 40X [25]. This is an attempt to maintain a physiological ratio between the two. Two concentrations of Trolox were examined since vitamin E in plasma and tissue spaces is apt to vary more than LDL or fibrinogen. These were 0.5 and 2 umol/l. In experiments where the combination of Trolox and fibrinogen was compared with Trolox and vitamin C the concentration of vitamin C was adjusted to give a lag phase similar to 50 mg/l of fibrinogen. This turned out to be near 6 umol/l vitamin C.

We notice that the prooxidant effect by Trolox was lost after a few months of LDL storage at −70°C. The effect was renewed when a new preparation of LDL was obtained.

When fibrinogen or fibrinogen with Trolox was added to the reaction mixture at 40 min, there was an abrupt increase in absorbance that then changed into a smooth curve. A series of experiments comparing the addition of fibrinogen to the LDL solution with and without copper and to buffer without copper or LDL indicated that the abrupt shift was due to the absorbance of fibrinogen at 234 nm and was unrelated to any change from oxidation. The calculated blank showed an absorbance change of 0.02–0.03 (not shown). The same phenomenon occurred when fibrinogen with Trolox was added. We adjusted the baselines to account for this absorbance change in the figures shown. The other antioxidants did not show more than a 0.02–0.03 absorbance change at 234 mm when added at 40 min and, therefore, required no baseline adjustment.

Calculations and statistics

The lag phase time in minutes for each antioxidant or combination was normalized by subtracting it from the average lag phase absorbance in the cuvets containing lipoprotein and copper without antioxidant to give a lag phase difference or Δ ([lag phase with antioxidant(s), copper and lipoprotein] − [lag phase

with copper and lipoprotein only]). A measure of cooperation or synergy was calculated by dividing the lag phase difference from the combination of two antioxidants together in the reaction solution by the sum of the lag phase difference of each individual antioxidant alone (Δ combination/Δ Trolox alone +fibrinogen alone or Trolox alone+vitamin C alone). We considered a combined effect greater than 1.2 (20%) as a synergistic effect, while we considered effects between 1 and 1.2 as being only additive.

Statistical analysis was performed using JMP (SAS Institute, Cary, NC). Mann Whitney using a two-tail test was used to obtain *p*-values.

Results

Antioxidant condition

In these experiments all antioxidants were added to the lipoprotein–copper solution at zero time. Under these conditions all of the antioxidants elongated the lag phase beyond that of copper and lipoprotein without antioxidant.

In a series of experiments, we examined the effects of 50 mg/l fibrinogen alone and 0.5 or 2 umol/l Trolox alone or combinations of fibrinogen and Trolox together. Figure 1 shows a typical experiment with duplicates. For this particular experiment the Table within Figure 1 indicates that the effect of adding a combination of Trolox and fibrinogen showed a combined effect of about 1.2 or 20% greater than the sum of Trolox and fibrinogen added alone for duplicate measurements. As can be seen in Table I, for all experiments, the combined effects for the combination of two different concentrations of Trolox with fibrinogen varied between 1.04 and 1.2 for two experiments with duplicates in each (4 replicates), showing increases beyond the sum of each alone of about 4–20%. We consider this degree of effect essentially equal or additive, no substantial synergy.

In order to compare the cooperation between Trolox and fibrinogen with that of Trolox and vitamin C, we identified a concentration of vitamin C that gave a lag phase elongation similar to that of fibrinogen. Figure 2 shows the results of this experiment. It was concluded that 6 umol/l vitamin C and 50 mg/l fibrinogen each showed a lag phase of about 100 min.

In a series of experiments, we examined the effects of 6 umol/l vitamin C alone and 0.5 or 2 umol/l Trolox alone and with 6 umol/l vitamin C and Trolox together. Figure 3 shows a typical experiment in which the antioxidants were added at zero time.

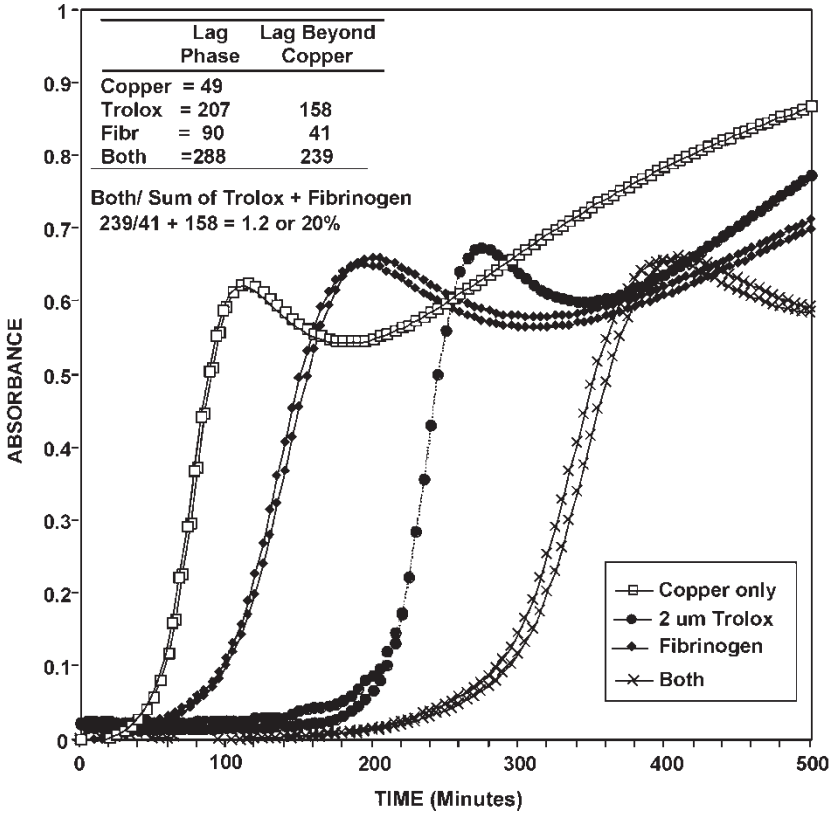

Figure 1. Typical experiment illustrating the effect of Trolox and fibringen added at zero time (antioxidant conditions). The reaction solution contained 30 mg/l LDL and 5 umol/l copper. The lag phases (min) were determined from the slopes of the duplicates. Lag phases were normalized by subtraction from the samples containing only LDL and copper. See the text for details and Table I for the results of all similar experiments that indicated the effects of Trolox and fibrinogen together showed a small effect that was about additive to the sum of each alone. Each anitoxidant or combination was performed in duplicate and the duplicate points are shown.

Table I. Lipoprotein protection by Trolox supplemented by fibrinogen or vitamin C under antioxidant-conditions (zero time).

Antioxidant	Concentration	Combined effect of duplicates	Average effect from combination	Lag phase (min) (Antioxidant—copper)
Trolox*	0.5 umol/l	From zero time		26
Fibrinogen*	50 mg/l			40
Both*		1.12		74
Trolox*	0.5 umol/l			39
Fibrinogen*	50 mg/l			59
Both*		1.09	1.11*	107
Trolox*	2 umol/l	From zero time		133
Fibrinogen*	50 mg/l			34
Both*		1.04		174
Trolox*	2 umol/l			158
Fibrinogen*	50 mg/l			41
Both*		1.2	1.08*	239
Trolox*	0.5 umol/l	From zero time		82
Vitamin C*	6 umol/l			17
Both*		1.43		142
Trolox*	0.5 umol/l			20
Vitamin C*	6 umol/l			47
Both*		2.28	1.86*	153
Trolox*	2 umol/l	From zero time		210
Vitamin C*	6 umol/l			40
Both*		1.64		410
Trolox*	2 umol/l			212
Vitamin C*	6 umol/l			18
Both*		1.82	1.73*	420

*All controls, antioxidants and combinations were performed in duplicate for a total of 4 replicates at each concentration and the average effect is for 4 replicates.

For this particular experiment the Table within Figure 3 indicates the effect of adding vitamin C and Trolox in combination as compared to each alone was about 1.64, or a synergy of 64% for the average of the duplicates. As can be seen in Table I effects for the combination of two different concentrations of Trolox with vitamin C for all experiments gave changes that varied between 1.43 and 2.28, reflecting synergies between about 43 and 128% for the 4 replicates in two experiments. This level of cooperation indicates that the combination showed a substantial synergistic effect over that of the sum of the two antioxidants measured separately.

Prooxidant condition

When low molecular weight antioxidants are added to the lipoprotein copper mixture, in a delayed manner, after the oxidation reaction has begun, many low molecular weight antioxidants behave as prooxidants [10,11,16]. When Trolox was added to LDL between 35 and 65 min after initiation of the oxidation reaction, it behaved as a prooxidant [16]. Preliminary experiments in our laboratory indicated that a 40 min delay produced a similar prooxidant effect at concentrations of both 0.5 and 2 umol/l Trolox.

Figure 4 shows a typical experiment in which Trolox and fibrinogen were added to the reaction solution after a delay of 40 min separately and in combination. For this experiment it can be seen in the table at the bottom of the figure that Trolox showed a prooxidant effect of −7 for the average of the duplicates, in that the lag phase was shorter than that in the cuvet containing copper and lipoprotein without antioxidant. But when fibrinogen was added in combination, the prooxidant effect was eliminated and an effect of about 3.00 was observed. This reflects a synergy of 200%. As can be seen in Table II, synergies for the combination as compared to each antioxidant alone, under this condition, varied between 1.54 and 3.00 or 54 and 200% for the four replicates each at two levels of Trolox. Thus, the prooxidant effect of Trolox was eliminated when fibrinogen was present to give an over all combined antioxidant effect which was substantially greater than the sum of the individual components alone.

Figure 5 shows a typical experiment depicting the effect of delayed addition of Trolox and vitamin C each alone and a combination of the two together. It can be seen in the table at the top of the figure that, although Trolox showed a prooxidant effect, for the average of the duplicates, with the lag phase for Trolox being −22 min less than the copper control, vitamin C showed an antioxidant effect of 16 min. Moreover, in terms of minutes, the combination together showed a synergistic antioxidant effect with a delay of 57 min more than the copper control while

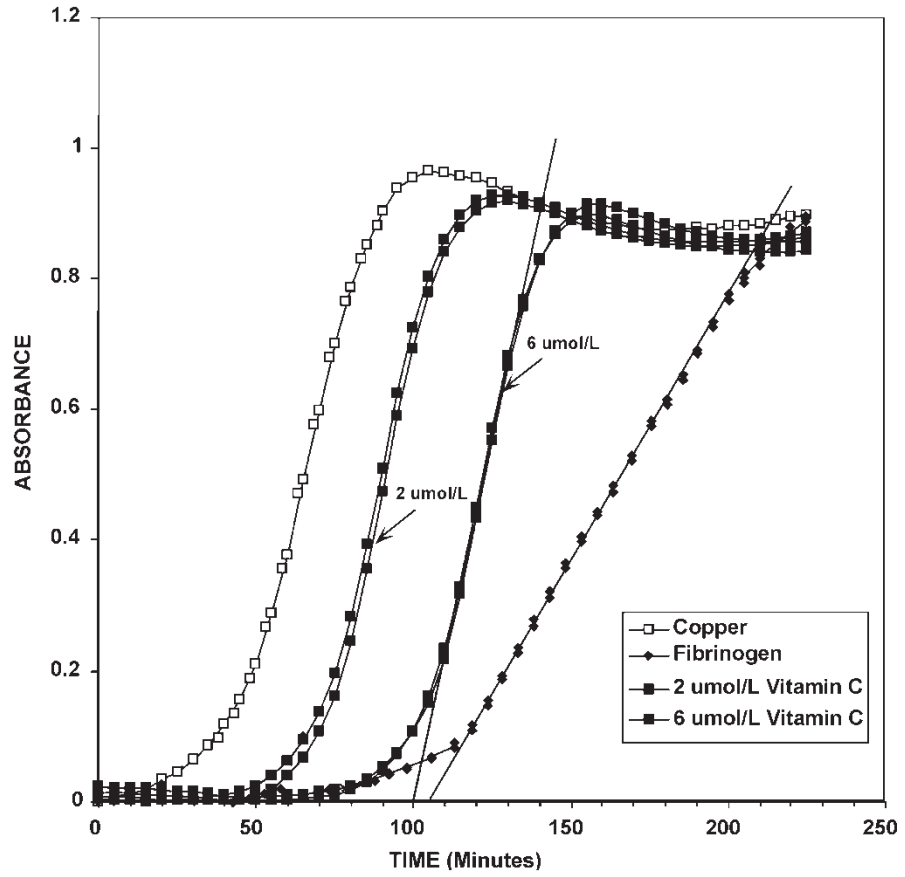

Figure 2. Determination of the concentration of vitamin C that produced a similar lag phase to 50 mg/l of fibrinogen. In this experiment, 2 and 6 umol/l vitamin C were tested. Otherwise conditions were similar to those in Figure 1. Each anitoxidant was performed in duplicate and the duplicate points are shown.

the sum of each alone showed an effect that was −6 min less than the copper control (16 − 22 = −6). Thus, there was a synergic effect of 63 min. These effects were similar when 0.5 umol/l Trolox was tested in combination with 6 umol/l vitamin C. Table III shows the results from a series of experiments where 4 replicates each at two concentrations of Trolox were measured.

Vitamin C has been shown to exhibit a prooxidant effect when added to a solution of LDL between 90 and 110 min after initiation of oxidation by copper [10]. With shorter delayed additions, vitamin C did not show a prooxidant effect but did show a reduced lag phase as compared to addition at zero time [10]. Similarly, we were not able to obtain a prooxidant effect at 40 min. Nevertheless, we also found a reduced lag phase at 40 min as compared to zero time for vitamin C. This is illustrated in Table IV where we derived the mean lag phase for 6 umol/l of vitamin C added at zero and 40 min from Tables I and III, and it can be seen that there was a significant difference between immediate and delayed addition ($p = 0.03$). On the other hand, the mean lag phase for 50 mg/l fibrinogen derived from Tables I and II, shown in Table IV, indicates that fibrinogen showed no significant difference between addition at 40 min and zero time ($p = 0.88$).

Discussion

We hypothesized that fibrinogen acts as a co-antioxidant that would supplement vitamin E and reduce its tendency for oxidizing lipoproteins. If this hypothesis is true *in vivo*, fibrinogen and similar molecules may play an important natural role in retarding arteriosclerosis and therapies based on the architecture of these molecules might prove important in retarding disease. In this study, we tested Trolox as our source of phenoxyl radical to determine whether or not this hypothesis should be pursued further. Based on comparisons of the lag phase derived from the reaction kinetics, we noticed that under antioxidant conditions fibrinogen supplemented Trolox in a way that showed little synergy above the additive effect of the two alone (4–20% shown in Table I and Figure 1), but under prooxidant conditions fibrinogen acted as a strong synergistic antioxidant with a combined effect between 54 and 200% greater than the sum of the antioxidants alone (Table II and Figure 4). Thus, we conclude that fibrinogen did act

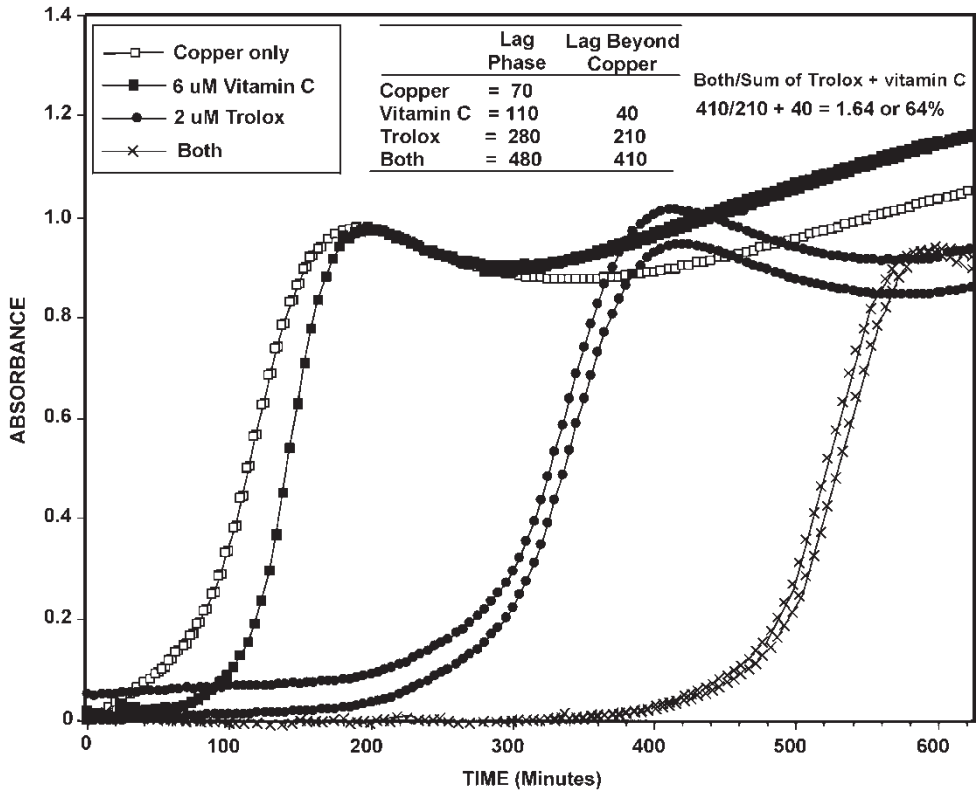

Figure 3. Typical experiment illustrating Trolox and vitamin C added at zero time (antioxidant conditions). Except for vitamin C substitution for fibrinogen, conditions were similar to that in Figure 1 with lag phases in minutes. See the text for details and Table I for the results of all similar experiments that indicated the effects of Trolox and vitamin C combined were synergistically much greater than the additive effects of each alone. Each antioxidant or combination was performed in duplicate and the duplicate points are shown.

as a supplemental co-antioxidant that eliminated the prooxidant effect of Trolox.

Still, these effects were in essence different than those seen between vitamin C and Trolox where a strong synergistic effect of between 43 and 128% was seen under antioxidant conditions (Table I and Figure 3). Recycling of Trolox by vitamin C has been well described [29] and can lead to a synergistic effect in protection of lipid oxidation that is similar to the behavior of alpha-tocopherol with vitamin C [18,19]. In cell or membrane systems vitamin C radical is depleted by disproportionation or further oxidation to dehydroascorbate that is coupled to mechanisms that replenish vitamin C [11,18,19,29]. This type of cooperation may be especially important in neutralizing the prooxidant behavior of radical forms *in vivo*.

If fibrinogen was recycling Trolox, we would have expected to see a more than additive synergistic effect under both antioxidant and prooxidant conditions similar to that seen with vitamin C, but for fibrinogen we only saw additive effects of between 4 and 20% under antioxidant conditions (Table I). Therefore, it is our conclusion that fibrinogen is not recycling Trolox but acting to directly quench the Trolox radical.

Nevertheless, under prooxidant conditions, vitamin C alone did show a reduced antioxidant effect exhibited by a shorter lag phase as compared to under antioxidant conditions (Table IV). On the other hand, fibrinogen did not show either the prooxidant effect of Trolox (Figure 4), nor did fibrinogen exhibit a prooxidant tendency towards a reduced lag phase as vitamin C did (Table IV). This data supports our hypothesis that fibrinogen does not support prooxidation under either condition.

Given the potential pro and antioxidant abilities of acute phase proteins and their easy access to interstitial spaces and plaque tissue, it seems important to better understand their role in atherosclerosis. We speculate that in physiological quantities fibrinogen does not exhibit a reactive radical form although more study is needed to confirm this. Although, it is known that albumin exhibits radical forms [30], it seems unlikely that natural albumin with bilirubin would do this since the bilirubin should deplete the albumin radicals while basic and aromatic amino acids in the globular portion of albumin may serve to bleed off bilirubin radicals. The unique structure of fibrinopeptides and globulin in fibrinogen may provide for a similar antioxidant mechanism. If some acute phase proteins act only as antioxidants under all conditions, they may have a potential as universal antioxidants for stabilizing systems of antioxidants under prooxidant conditions where

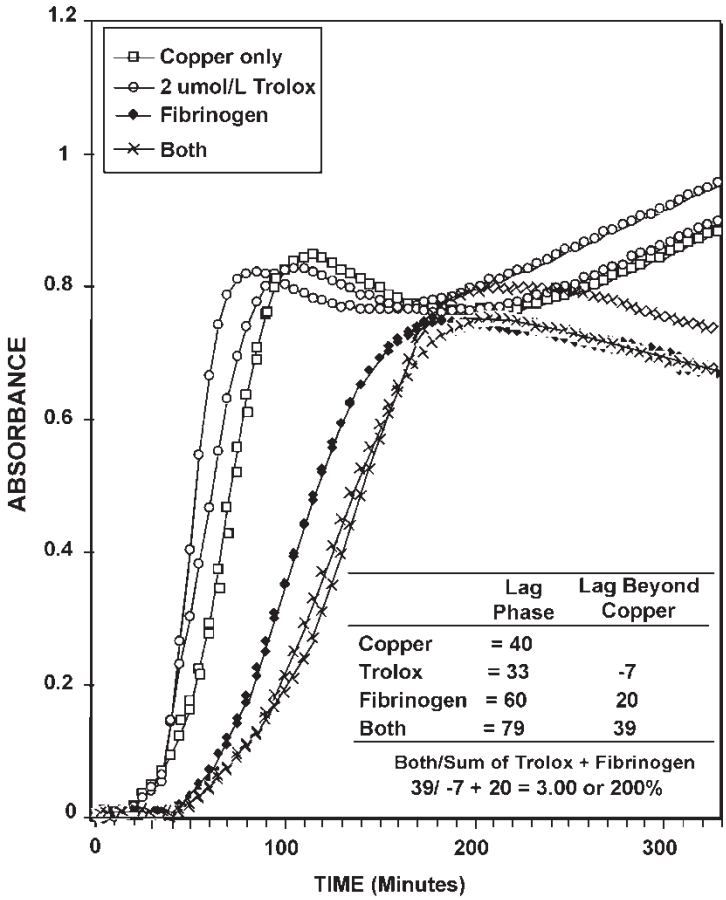

Figure 4. Typical experiment illustrating the effect of Trolox and fibrinogen added with a 40 min delay (prooxidant conditions). The reaction solution contained 30 mg/l LDL and 5 umol/l copper at zero time and the antioxidants were added later. The lag phase (min) was determined as in Figure 1. See the text for details and Table II for the results of all similar experiments that indicated the effects of Trolox and fibrinogen combined showed a large synergy as compared to the sum of each alone. Each anitoxidant or combination was performed in duplicate and the duplicate points are shown.

small molecules exhibit radical forms, without showing any prooxidant behavior themselves.

Weaknesses of this study are that for simplicity in this initial study, we used Trolox in a cell free system rather than natural vitamin E in a more biological system.The redox potential of Trolox is very similar to alpha-tocopherol [31,32] and, as discussed above, the two behave very similar in exhibiting a prooxidant effect and in being recycled by vitamin C [16,29].

Nevertheless, although the mechanisms by which co-antioxidants react biochemically with the phenolic group of Trolox and physiological forms of vitamin E

Table II. Lipoprotein protection by Trolox supplemented by fibrinogen under prooxidant conditions (40 min delay).

Antioxidant	Concentration	Combined effect	Average effect from combination for 4 replicates	Lag phase (min) (Antioxidant—copper)
Trolox*	0.5 umol/l	At 40 min		−11
Fibrinogen*	50 mg/l			49
Both*		1.97		75
Trolox*	0.5 umol/l			−13
Fibrinogen*	50 mg/l			39
Both*		2.50	2.23*	66
Trolox*	2 umol/l	At 40 min		−7
Fibrinogen*	50 mg/l			20
Both*		3.00		39
Trolox*	2 umol/l			−12
Fibrinogen*	50 mg/l			49
Both*		1.54	2.16*	57

*All controls, antioxidants and combinations were performed in duplicate for a total of 4 replicates at each concentration. Thus, the average effect is for 4 replicates.

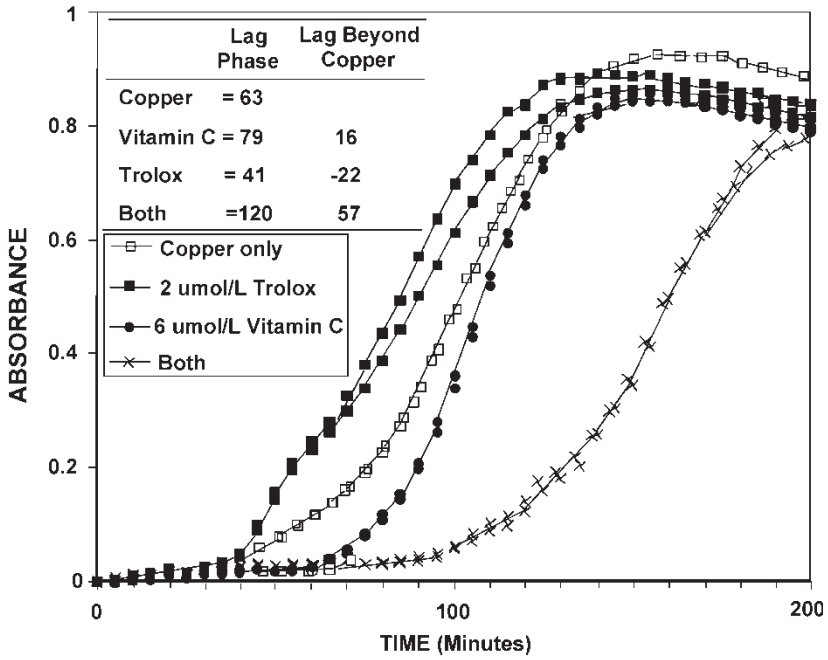

Figure 5. Typical experiment illustrating the effect of Trolox and vitamin C added at 40 min (prooxidant conditions). The reaction solution contained 30 mg/l LDL and 5 umol/l copper from zero time and the antioxidants were added later. The lag phase (min) was determined as in Figure 1. See the text for details and Table III for the results of all similar experiments that indicated Trolox added alone caused a prooxidant effect while the two in combination caused an antioxidant effect. Each anitoxidant or combination was performed in duplicate and the duplicate points are shown.

are similar, tocopherols are bound within the LDL while Trolox is in the aqueous phase. Therefore, it remains unclear whether fibrinogen can act as a physiological co-antioxidant with tocopherols. But, since natural albumin with bilirubin appears to act as a co-antioxidant to recycle the phenoxy radical, and the globular portion of fibrinogen shows less negative charge than albumin, it seems likely that fibrinogen would be another protein that can act as a co-antioxidant with vitamin E. Based on the biochemical results obtained in this study, it seems worthwhile to pursue our hypothesis further by measuring interactions between alpha-tocopherol and fibrinogen in LDL or membrane preparations under pro- and antioxidation conditions to confirm that these effects occur with tocopherols.

In summary and conclusion, we have shown that fibrinogen acts as a co-antioxidant in conjunction with Trolox in protecting lipoproteins from oxidation, only additively under antioxidant conditions, but synergistically under prooxidant conditions. The effects under antioxidant conditions were different from those of vitamin C, suggesting that fibrinogen quenches the prooxidant effects of Trolox radical rather than recycling it. Unlike Trolox, alpha-tocopherol, vitamin C, and many other low molecular weight antioxidants,

Table III. Lipoprotein antioxidant protection by Trolox supplemented by vitamin C under prooxidant conditions (40 min delay).

Antioxidant	*N*	Concentration	Lag phase (min)	Lag phase—copper (min)	Increase of combination over the sum of each alone (min)
Trolox	2	0.5 umol/l	51	−10	
Vitamin C	2	6 umol/l	69	8	increase = 19
Both	2		78	17	sum of each = −2
Trolox	2	0.5 umol/l	50	−5	
Vitamin C	2	6 umol/l	68	13	increase = 14
Both	2		77	22	sum of each = 8
Trolox	2	2 umol/l	40	−15	
Vitamin C	2	6 umol/l	63	8	increase = 45
Both	2		93	38	sum of each = −7
Trolox	2	2 umol/l	41	−22	
Vitamin C	2	6 umol/l	79	16	increase = 63
Both	2		120	57	sum of each = −6

N = Number of replicate observations in each experiment. Sum = (Lag phase of Trolox—copper)((Lag phase of vitamin C—copper). Increase (or synergy on minutes) = (Lag phase of both—copper) − sum.

Table IV. Comparison of antioxidant effect of fibrinogen and vitamin C under antioxidant and prooxidant conditions.

Substance	Time of addition (min)	$N^{\star}$	Mean lag[†] phase (min)	Range[†]of lag phases	Z	p
Fibrinogen (50 mg/l)	0	4	43.5	34–59		
Fibrinogen (50 mg/l)	40	4	39.25	20–49	0.145	0.88
Vitamin C (6 umol/l)	0	4	30.5	17–47		
Vitamin C (6 umol/l)	40	4	11.2	8–16	−2.2	0.03

⋆ Total number of observations from 2 experiments.
† From Tables I–III.
Zero minutes = antioxidant condition; 40 min = prooxidant conditions.

fibrinogen did not appear to exhibit prooxidant tendencies in a prooxidant environment. The results suggest that fibrinogen may add stabilization to systems of antioxidants under prooxidant conditions. The importance of acute phase protein interactions in oxidation and atherosclerosis is poorly understood and needs further study. Continued study of these processes in more physiological systems seems worthwhile.

Acknowledgements

This work was supported by the Department of Veterans Affairs, Louisville, KY.

References

[1] Zaman AG, Helft G, Worthley SG, Badimon JJ. The role of plaque rupture and thrombosis in coronary artery disease. Atherosclerosis 2000;149:251–266.
[2] Third report of the National Cholesterol Education Program (NCEP) Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report. ATP III final report. Circulation 2002; 106:3145–3234.
[3] Jensen LO, Thayssen P, Pedersen KE, Stender S, Haghfelt T. Regression of coronary atherosclerosis by simvastatin: A serial intravascular ultrasound study. Circulation 2004;110: 265–270.
[4] Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: An overview. Free Radic Biol Med 2000;28:1815–1826.
[5] Neuzil J, Weber C, Kontush A. The role of vitamin E in atherogenesis: Linking the chemical, biological and clinical aspects of the disease. Atherosclerosis 2001;157: 257–283.
[6] Kritharides L, Stocker R. The use of antioxidant supplements in coronary heart disease. Atherosclerosis 2002;164:211–219.
[7] Abudu N, Miller JJ, Attaelmannan M, Levinson SS. Vitamins in human arteriosclerosis with emphasis on vitamin C and vitamin E. Clin Chim Acta 2004;339:11–25.
[8] Maiorino M, Zamburlini A, Roveri A, Ursini F. Prooxidation role of vitamin E in copper induced lipid peroxidation. FEBS Lett 1993;330:174–176.
[9] Neuzil J, Thomas SR, Stocker R. Requirement for, promotion, or inhibition by alpha-tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation. Free Radic Biol Med 1997;22:57–71.
[10] Otero P, Viana M, Herrera E, Bonet B. Antioxidant and prooxidant effects of ascorbic acid, dehydroascorbic acid and flavonoids on LDL submitted to different degrees of oxidation. Free Radic Res 1997;27:619–626.
[11] Bagnati M, Perugini C, Cau C, Bordone R, Albano E, Bellomo G. When and why a water-soluble antioxidant becomes pro-oxidant during copper-induced low-density lipoprotein oxidation: A study using uric acid. Biochem J 1999;340:143–152.
[12] Giles GJ, Tasker KM, Jacob C. Hypothesis:The role of reactive sulfur species in oxidative stress. Free Radic Biol Med 2001;31:1279–1283.
[13] Smith C, Mitchinson MJ, Aruoma OI, Halliwell B. Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. Biochem J 1992;286:901–905.
[14] Niu X, Zammit V, Upston JM, Dean RT, Stocker R. Coexistence of oxidized lipids and alpha-tocopherol in all lipoprotein density fractions isolated from advanced human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 1999;19:1708–1718.
[15] Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: Combinations to avoid. Radiat Res Albertini R, Abuja PM. Prooxidant and antioxidant properties of trolox C, analogue of vitamin E, in oxidation of low-density lipoproteins. Free Radic Res 1999;30:181–188 1996;145: 532–541.
[16] Albertini R, Abuja PM. Prooxidant and antioxidant properties of trolox C, analogue of vitamin E, in oxidation of low-density lipoproteins. Free Radic Res 1999;30:181–188.
[17] Thomas SR, Neuzil J, Mohr D, Stocker R. Coantioxidants make alpha-tocopherol an efficient antioxidant for low-density lipoprotein. Am J Clin Nutr 1995;62:1357S–1364S.
[18] Leung HW, Vang MJ, Mavis RD. The cooperative interaction between vitamin E and vitamin C in suppression of peroxidation of membrane phospholipids. Biochim Biophys Acta 1981;664:266–272.
[19] Winkler BS. Unequivocal evidence in support of the nonenzymatic redox coupling between glutathione/glutathione disulfide and ascorbic acid/dehydroascorbic acid. Biochim Biophys Acta 1992;1117:287–290.
[20] Bini A, Fenoglio Jr JJ, Mes, a-Tejada R, Kudryk B, Kaplan KL. Identification and distribution of fibrinogen, fibrin, and fibrin(ogen) degradation products in atherosclerosis. Arteriosclerosis 1989;9:109–121.
[21] Valenzuela R, Shainoff JR, DiBello PM, Urbanic DA, Anderson JM, Matsueda GR, Kudryk BJ. Immunoelectrophoretic and immunohistochemical characterizations of fibrinogen derivatives in atherosclerotic aortic intimas and vascular prosthesis pseudo-intimas. Am J Pathol 1992;141: 861–880.
[22] Halliwell B. Albumin—An important extracellular antioxidant? Biochem Pharm 1988;37:569–571.
[23] Neuzil J, Stocker R. Free and albumin-bound bilirubin are efficient co-antioxidants for alpha-tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation. J Biol Chem 1994;269:16712–16719.
[24] Olinescu RM, Kummerow FA. Fibrinogen is an efficient antioxidant. J Nutrit Biochem 2001;12:162–169.

[25] Kaplan IV, Att, aelmannan M, Levinson SS. Fibrinogen is an antioxidant that protects β-lipoproteins at physiological concentrations in an *in vitro* cell free system. Atherosclerosis 2001;158:455–463.

[26] Lupidi G, Angeletti M, Eleuteri AM, Tacconi L, Coletta M, Fioretti E. Peroxynitrite-mediated oxidation of fibrinogen inhibits clot formation. FEBS Lett 1999;462: 236–240.

[27] Maciejko JJ, Levinson SS, Markyvech L, Smith M. Evaluation of a new method for assaying apo A-I and apo B Lipoproteins by rate nephelometry. Clin Chem 1987;11:2065–2069.

[28] Kaplan IV, HobbsGA, Levinson SS. Simpler technique for measuring oxidative susceptibility by heparin affinity column isolation of lipoproteins. Clin Chim Acta 1999;283: 89–103.

[29] Guo Q, Packer L. Ascorbate-dependent recycling of the vitamin E homologue trolox by dihydrolipoate and glutathione in murine skin homogenates. Free Radic Biol Med 2000;29:368–374.

[30] Ostdal H, Davies MJ, Andersen HJ. Reaction between protein radicals and other molecules. Free Radic Biol Med 2002;33:201–209.

[31] Buettner GR. The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate. Arch Biochem Biophys 1993;200:535–543.

[32] Bergmann AR, Ramos P, Esterbauer H, Winklhofer-Roob BM. RRR-alpha-tocopherol can be substituted for by trolox in determination of kinetic parameters of LDL oxidizability by copper. J Lipid Res 1997;38:2580–2588.