ELSEVIER

Journal of Nutritional Biochemistry 12 (2001) 162–169

THE JOURNAL OF Nutritional Biochemistry

Fibrinogen is an efficient antioxidant

Radu M. Olinescu[a,b], Fred A. Kummerow[a,b,*]

[a]*The Burnsides Research Laboratory, Urbana, IL 61801 USA*
[b]*The Harlan E. Moore Heart Research Foundation, Champaign, IL 61820 USA*

Received 14 June 2000; received in revised form 29 September 2000; accepted 17 October 2000

Abstract

Fibrinogen has been included among the risk factors for vascular disease. Fibrinogen belongs with albumin, ceruloplasmin and transferrin to an acute phase protein group in the plasma. Albumin, ceruloplasmin and transferrin are already recognized as natural antioxidants. In the present study we used three different oxygen generating systems in order to test whether fibrinogen is able to act as an antioxidant in an in vitro system. We used 1) pyrogallol auto-oxidation, 2) the reaction catalysed by xanthine oxidase coupled with the reduction of ferricytochrome c and 3) chemiluminescence. We found that in a dose-dependent manner fibrinogen inhibited superoxide generation (pyrogallol and xanthine-xanthine oxidase reactions), ferrous ion oxidation and hydroxyl radical dependent degradation (of deoxyribose). Fibrinogen also inhibited LDL oxidation (copper and azo compound-induced), hydrogen peroxide oxidation and chemiluminescence produced by polymorphonuclear leukocytes. Fibrinogen, albumin, ceruloplasmin and transferrin act as a supplementary antioxidant defense mechanism against oxidative stress arising from inflammatory conditions.

Keywords: Fibrinogen; Antioxidants; Atherosclerosis; Oxygen free radicals

1. Introduction

Clinical [1–3] and epidemiological studies [4–8] have established that an elevated fibrinogen (FB) concentration in the plasma is an independent risk factor for vascular disease. FB and its degradation products are basic components of the atherosclerotic plaque and blood clots in veins [9,10].

Two recent reviewers [11,12] have discussed the hypothesis that low density lipoprotein (LDL) must be oxidatively modified to oxidized LDL (ox-LDL) in order to trigger the pathological events leading to atherosclerosis. An increase in ox-LDL, found in patients with coronary artery disease [13,14] and in patients subjected to heart transplants [15], has been credited with an important role in the initiation and progression of atherosclerosis [16,17]. The increase in ox-LDL correlated not only with the extent of coronary artery stenosis but also with the development of the stenosis [18]. These oxidation products induced damage to cellular membrane, caused loss of cell viability [19], inhibited wound-healing response of vascular endothelial cells [20] and produced an increase in cytosolic calcium [21]. This increase was associated with irreversible morphological changes. Ox-LDL also induced activation of endothelial recruitment of leukocytes, enhancement of macrophage cytokine production and stimulation of smooth muscle cell proliferation [22]. The survival of cells was significantly reduced [23] when ox-LDL was added into a culture medium.

In vitro studies indicate that oxidation in the plasma is challenged by a host of defense mechanisms [24] including albumin [25]. Albumin inhibits both metal and peroxyl radical mediated lipid peroxidation of LDL [26]. We found that free and albumin-bound bilirubin at the physiological concentration of the bile pigment in blood plasma could greatly inhibit metal-catalyzed oxidation of LDL. This was shown by reduced thiobarbituric acid reactivity, smaller or lack of shifts in electrophoretic mobility, less apo B fragmentation and decreased amount of cholesterol oxidation products as detected by gas chromatography [26].

We found in cardiac catheterized patients with proven angina that the concentration of acute phase proteins and lipid oxidation products were significantly higher than in the plasma of age and sex matched non-smoking, non-cardiac catheterized individuals without apparent coronary heart disease [27]. In the present study we used fibrinogen as an antioxidant to inhibit reactive oxygen species in an in vitro system as recommended by Halliwell [28,29]. We used

* Corresponding author. Tel.: 217-333-1806; fax: 217-333-7370.
E-mail address: fkummero@uiuc.edu (F.A. Kummerow).

0955-2863/01/$ – see front matter
PII: S0955-2863(00)00147-9

pyrogallol as an oxygen generating system and also used the reaction catalysed by xanthine oxidase coupled with the reduction of ferricytochrome c and chemiluminescence produced by polymorphonuclear leukocytes.

2. Materials and methods

Human fibrinogen (FB), grade 4, from Kabi AB was dialyzed for 6 hours at room temperature against 50 μM Tris-HCl buffer, pH 7.3. The final solution was divided into aliquots and stored at −20°C until use. According to the manufacturer, this commercial FB contains only trace amounts of factor XIII and no more than 0.9% by weight of plasmin-related material. In preparation for use, a frozen aliquot of FB solution was first thawed to room temperature, diluted to the desired concentration by using the same buffer and then heated to 37°C to dissolve any residual. The FB concentration of the stock solution was determined by using the molar absorptivity of the protein at 280 nm, 5.12×10^5 L mol^{-1} cm^{-1}. The remaining biochemical compounds were purchased from Sigma Chemical Co. (St. Louis, Mo). The 2,2′-Azobis-(2-amidinopropane) hydrochloride (AAPH) was obtained from Wako (Chicago, Ill). Procedures were carried out in plastic or acid-washed glassware, and solutions were prepared in Chelex 100 resin-treated glass-twice distilled water. In choosing different methods for assaying anti-oxidative properties of FB and other compounds, we followed the recommendations made by Halliwell [28,29].

2.1. Superoxide anion oxygen scavenging

Due to several interfering factors [29], we used two methods to assay oxygen scavenging properties. The first involved pyrogallol auto-oxidation where the increase in absorbance values at 420 nm (A_{420}) was due to the generation of superoxide anion as described by Lappena et al. [30]. The second involved the generation of O_2 during a xanthine-xanthine oxidase reaction coupled with the reduction of ferricytochrome c under the conditions described by Goldstein et al. [31]. In both methods mean rates as A_{420} nm/min and A_{550} nm/min respectively were used.

2.2. Fe^{2+} auto-oxidation

Spontaneous auto-oxidation of Fe^{2+} as a source of perferryl ion ($Fe^{3+} \cdot O_2^{-}$) was measured as described by Tadolini et al. [32] using 100 μM $FeCl_2$ in Mops buffer, pH 7.5. We also used the competitive action of cumene hydroperoxide as a source of peroxides.

2.3. Formation of hydroxyl radical

For the formation of hydroxl radicals ($OH^{\cdot}$) we used the reaction of deoxyribose and Fe^{3+} in the presence of hydrogen peroxide according to Gutteridge and Wilkins [33]. After the reaction with ferric chloride (1 mM), deoxyribose (7.5 mM) and hydrogen peroxide (10 mM), the damage to deoxyribose was measured by using the thiobarbituric acid reaction [33].

2.4. Azo compound-initiated oxidation of LDL

AAPH is a water soluble azo compound that thermally decomposes leading to formation of aqueous peroxyl radicals at a constant rate. During the reaction, the samples were incubated at 37°C. The AAPH study was performed under the conditions described by Gaziano et al. [34]. These conditions include isolation of LDL, removal of natural antioxidants by column gel filtration, followed by density gradient ultracentrifugation at 7°C at 43,000 rpm for 24 hours in a Beckman L-350 ultracentrifuge. Final preparations were dialyzed extensively against 0.15 mol/L NaCl, 20 mmol/L sodium phosphate and 2 mmol/L EDTA pH 7.4 at 4°C. Protein concentrations were determined by the method of Marcat and Gerbant [35]. The final concentration was adjusted to 50 μg/ml.

2.5. Hydrogen peroxide (H_2O_2) scavenging

The possible scavenging effects on H_2O_2 were investigated by using a modified guaiacol-myeloperoxidase system as described by Lapenna et al. [30]. Briefly, hydrogen peroxide in control and in FB-containing samples was assayed spectrophotometrically by measuring the formation of a brown chromogen in 1.0 ml reaction mixtures containing 50 mM KH_2PO_4/KOH buffer, pH 7.0, and 50 μl of guaiacol solution (7 mM) and myeloperoxidase, 53 mU/ml. The reaction was triggered by the addition of 120 μM hydrogen peroxide. The absorption of the brown chromogen was measured at 470 nm.

2.6. Chemilumeniscence produced by activated polymorphonuclear leukocytes

Isolation of polymorphonuclear leukocytes (PMNL) from healthy blood donors was performed according to the procedure described by Trush [36]. The final preparation was adjusted to 1×10^7 cells/ml, of which 70% were neutrophils, in Hanks' Balanced Salt Solution containing 0.1 mM calcium and magnesium. The reaction mixture contained 0.1 ml PMNL (1.10^6 cells), 0.1 ml luminol (100 μM) and 0.8−0.7 ml Hanks medium. For control only PMNL and luminol were used while for the samples 0.1 ml opsonized zymosan (1 mg) was added to trigger the chemiluminiscence (CL). CL emission was measured in a Beckman liquid scintilator counter by using a special single photon counting program. A cell-free chemiluminescent experiment was also done by measuring CL decay rate of FB and other known antioxidants. The reaction mixture contained varied concentrations of purified FB or an antioxidant and 100 μM of luminol, phosphate buffer 100 mM

Table 1
Inhibition (%) of superoxide generation by fibrinogen and some known antioxidants in pyrogallol autooxidation and ferricytochrome c reduction in the xanthine oxidase-hypoxanthine (XO − HX) system

Compounds	Pyrogallol	XO − HX
Control (no added compounds)	100	100
Superoxide dismutase (0.1 U/ml)	18 ± 3*	17 ± 2*
Superoxide dismutase (0.5 U/ml)	32 ± 4*	26 ± 3*
Superoxide dismutase (1 U/ml)	64 ± 4**	53 ± 5**
Trolox (0.5 mM)	39 ± 4**	45 ± 6**
Fibrinogen 3 μM	12 ± 2*	7 ± 1
Fibrinogen 6 μM	19 ± 3*	15 ± 2*
Fibrinogen 12 μM	29 ± 4*	20 ± 3*
Fibrinogen 18 μM	35 ± 6*	29 ± 5*

Superoxide anion was generated using 0.4 mmol L^{-1} pyrogallol in 2.5 ml of 50 mmol/L Tris-HCl buffer, pH 8.2 containing 1 mmol L^{-1} diethylene triamine pentacetic acid. For the reduction of ferricytochrome, the reaction mixture contained 3.0 mU of xanthine oxidase, 0.1 mmol hypoxanthine, 5 μmol ferricytochrome c in 3.0 ml of 0.05 M potassium phosphate buffer, pH 7.8. Data were transformed for convenience from the mean rates of pyrogallol autooxidation (A_{420} nm/min) or ferricytochrome c reduction (A_{550} nm/min) where $n = 5$. The variation in original values were less than 10%. * Significantly different ($P < 0.05$) and ** ($P < 0.01$) from individual controls (no test compound added). Control—100% inhibition.

at pH 7.4 in a final volume of 3 ml. The addition of H_2O_2 (10 mM) triggered the generation of CL [37].

2.7. Statistical analysis

Data were indicated as means ± SD of five different determinations. All calculations were performed by using a statistical package from BMDP Statistical Software Inc. Differences between variants were analyzed by the Student's *t* Test for unpaired data. $P < 0.05$ was regarded as statistically significant. We used molar expression of FB concentration as well as mg/ml as commonly used in order to compare its effects with other known antioxidants.

3. Results

3.1. Superoxide anion oxygen scavenging

Our results demonstrate that FB inhibits oxygen free radicals in a dose dependent manner. As shown in Table 1, purified FB at physiological concentration exhibited almost the same inhibitory properties in two oxygen generating systems. As Halliwell pointed out [28,29] the measurement of an oxygen scavenging property of a compound might produce erroneous conclusions. We used as an oxygen generating systems a pyrogallol test [31] and used the reaction catalyzed by xanthine oxidase coupled with the reduction of ferricytochrome c and nitroblue tetrazolium to study the effects of purified fibrinogen as an antioxidant in vitro. No interaction was observed between FB and cytochrome c or xanthine oxidase. Inhibitory effects of FB at physiological levels (6–12 μM) could be compared with a low level of superoxide dismutase whereas increased concentrations that occur in inflammatory conditions (18 μM) matched those of Trolox, a water soluble derivative of α- tocopherol (Table 1). The moderate, antioxidant effect of FB (at the physiological concentration of 12 μM) towards the superoxide radical can be expected to be more effective under inflammatory conditions which increased FB concentrations.

The stimulatory effect of Fe^{2+} ions on lipid peroxidation is universally recognized [32,33,37,38]. Fe^{2+} acts in all stages of lipid peroxidation and could be regarded an antioxidant (Fig. 1). FB was able to inhibit Fe^{2+} oxidation at physiological levels in a concentration-dependent manner. The inhibitory effects of FB increased with its concentration.

In Fig. 1B, the action of FB took place in a more complex situation, for example, when Fe^{2+} oxidation was influenced by the presence of preformed peroxides such as cumene hydroperoxides (cumene-OOH). In which case cumene-OOH rapidly oxidized $FeCl_2$ while FB action was less significant but still effective at a higher concentration. A MEDLINE search indicated no previous studies showing an interaction between Fe and FB. Our results indicate a significant binding of iron ions with FB thus inhibiting their oxidation.

3.2. Hydroxyl radical ($OH^{\cdot}$) scavenging

The deoxyribose test has been considered the most suitable means for detecting scavenging properties towards the OH radical [38]. This test shows that deoxyribose oxidation results in the formation of aldehydic compounds that are able to react with thiobarbituric acid. This test performed without EDTA can also give information regarding iron-binding properties. As seen in Table 2, FB at its physiological concentration is able to scavenge significantly the OH radical. Albumin is also able to scavenge the OH radical similar to Trolox and ascorbate [39]. The inhibitory effect of FB increases with a concentration similar to albumin. The presence of additional EDTA did not significantly increase the inhibitory effect of FB.

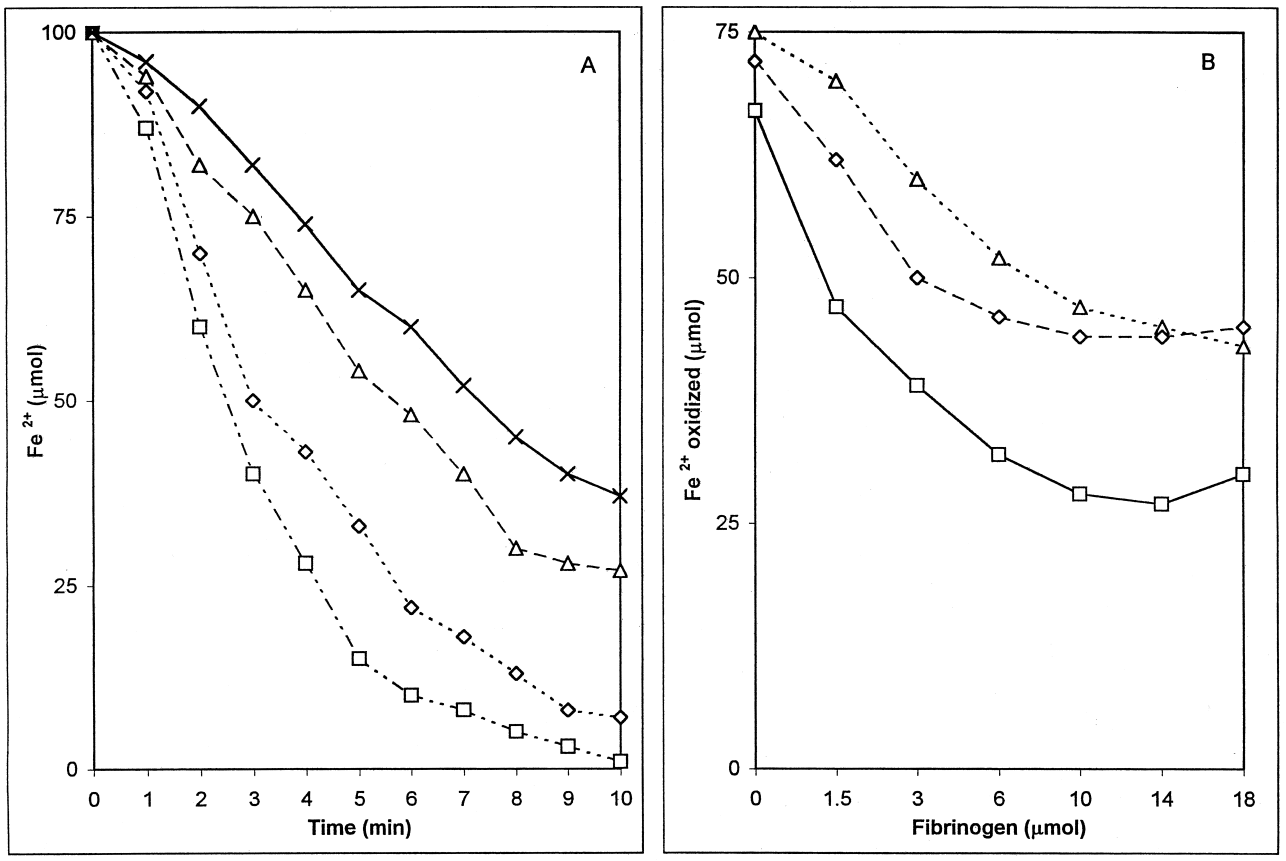

Fig. 1. Effects of fibrinogen on Fe^{2+} oxidation: A. Time course of Fe^{2+} oxidation in absence (□----□) or in presence of 500 μg/ml (1.3 μM) (◇----◇), 1 mg/ml (6 μM) (△----△ and 3 mg/ml (18 μM) (X----X) purified fibrinogen. B. Inhibition of Fe^{2+} oxidation as a function of FB concentration in absence (□——□) or presence of 10 μM (◇----◇) and 30 μM (△----△) of cumene hydroperoxide.

3.3. Copper and azo-compound initiated oxidation of LDL

Fibrinogen plays an essential role in the process of blood coagulation as well as in plaque formation [40,41]. If Ox-LDL triggers the formation of plaques within blood vessels, an interaction between FB and oxLDL is likely to occur. The azo-compound-initiated oxidation of LDL appeared suitable for this study. The inhibitory properties of FB were found in both Cu^{2+} and AAPH-induced LDL oxidation (Table 3). In both tests FB inhibited LDL oxidation significantly in concentrations at the upper limit of physiological level of approximately 12 μM. In these tests, FB seemed to be as powerful an antioxidant as Trolox or ascorbate when compared to the control ($P < 0.05$). Albumin also inhib-

Table 2
Effects of some compounds on deoxyribose degradation by iron(III), according to Gutteridge method [16]

Additions	Rate of formation of thiobarbituric reactivity from deoxyribose 1 h	
	A_{532}	% inhibition
Blank (H_2O_2 + deoxyribose)	0.083 ± 0.003	—
(Fe^{3+} + deoxyribose)	0.085 ± 0.005	—
Control (Fe^{3+} + H_2O_2 + deoxyribose)	0.534 ± 0.08	—
+ Catalase (0.1 mg/ml)	0.073 ± 0.003	97
+ Superoxide dismutase (0.1 mg/ml)	0.416 ± 0.03	22
+ Albumin (0.2 mg/ml; 3 μM)	0.454 ± 0.04	13
+ Albumin (2 mg/ml; 30 μM)	0.375 ± 0.02	30
+ Fibrinogen (1 mg/ml; 3 μM)	0.579 ± 0.05	—
+ Fibrinogen (2 mg/ml; 6 μM)	0.342 ± 0.04	36
+ Fibrinogen (4 mg/ml; 12 μM)	0.314 ± 0.03	41

All concentrations shown are final reaction concentrations. The % inhibition was calculated after subtraction of appropriate blanks. For the control system, the final concentrations were 0.1 mM (ferric ions), 1.0 mM (hydrogen peroxide) and 2 mM (deoxyribose).

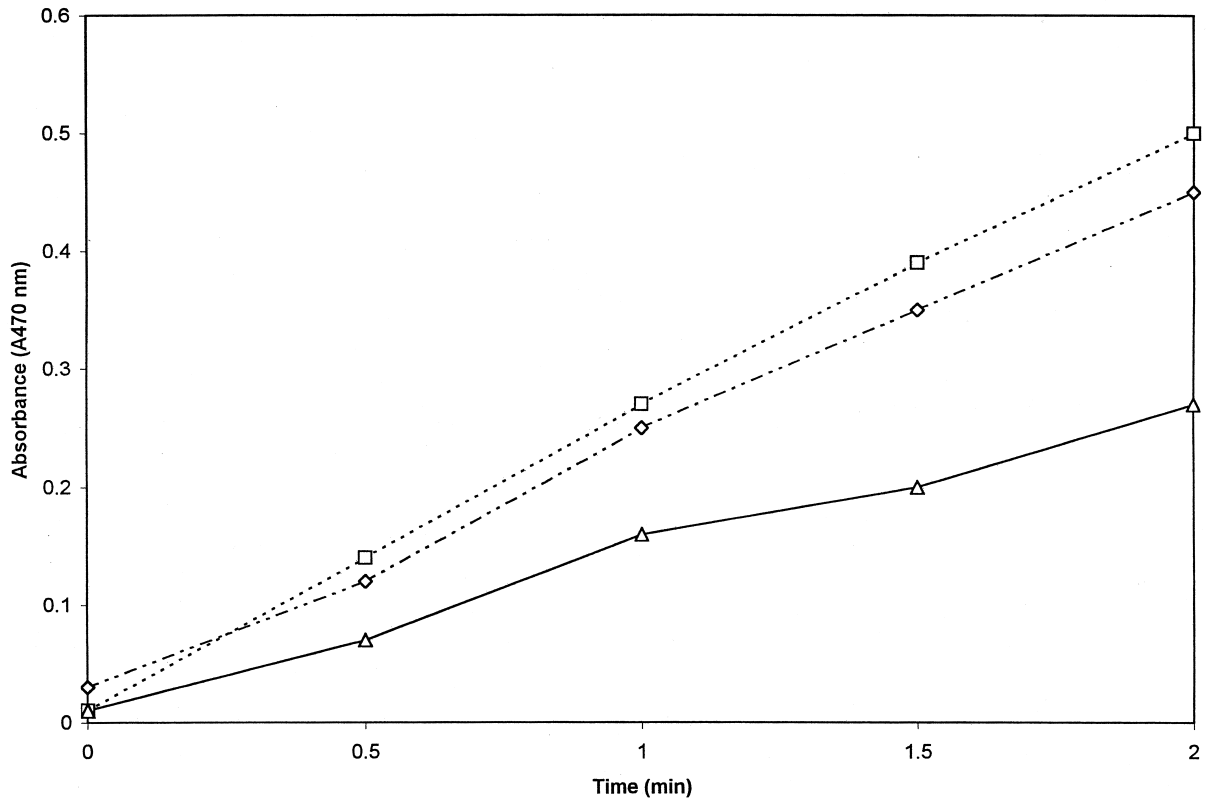

Fig. 2. Myeloperoxidase (MPO)-hydrogen peroxide-mediated oxidation of guaiacol in a cell-free system. No change in absorbance at 470 nm was observed when H_2O_2 or MPO (53 mU/ml) were added to the FB solutions (but no guaiacol). Guaiacol (7 mM) and MPO were incubated in chloride-free phosphate buffer (50 mM, pH 7.0) at 37°C for 5 min in the absence (□----□) and in the presence of 2 mg/ml (◇----◇) or 4 mg/ml (△——△ fibrinogen.

ited LDL oxidation significantly [26,27]. Fibrin, fibrinopeptide A, fibrinogen degradation product X or cumene hydroperoxide did not inhibit LDL oxidation.

3.4. Hydrogen peroxide-peroxidase-mediated oxidation

Since myeloperoxidase is one of the systems that function in leukocytes during phagocytosis, we studied a possible action of FB in a cell-free experiment. As presented in Fig. 2, purified FB can also inhibit the peroxidase-H_2O_2-mediated oxidation of guiacol. As shown in previous tests [28,29], FB inhibits oxidation of guiacol only at the upper limit of the physiological level of 12 μM.

3.5. Chemiluminescence studies

Chemiluminescence (CL) is a useful method for measuring the antioxidant property of compounds that are being recommended for the study of new anti-inflammatory drugs [42]. CL produced by activated PMNL is most suitable for this purpose. FB clearly modifies the CL produced by opsonized zymosan-induced activation of human polymorphonuclear leukocytes (Fig. 3). This inhibition was strongly related to FB concentration. Albumin also inhibited CL emission produced by activated PMNL. Our data are in agreement with Higazi et al. [43] on the inhibition of neutrophil activation by FB. FB decreased the chemotactic activity and oxygen consumption of neutrophils in a dose dependent manner. We also found an inhibitory action of albumin on CL produced by activated PMNL. The dose-dependent manner inhibition of CL previously shown by our data might suggest a modulatory function of FB on PMNL activation as already suggested by Higazi [43]. A cell-free CL technique where the oxygen free radicals were produced by a reaction between luminol and hydrogen peroxide [36] showed that FB inhibited CL emission in a dose dependent manner as did Trolox and ascorbate (Fig. 4). Comparing the rates of CL emission revealed that FB exhibited a significant inhibitory effect only at the upper limit of physiological range (12 μM) and towards higher pathological levels (18 μM). The specific CL kinetic curve of FB matched that of albumin and other proteins having exposed aromatic amino acid residues [43].

4. Discussion

A defense mechanism against the marked rise of oxygen free radicals released by activated leukocytes during inflammatory conditions may explain the increased fibrinogen concentration in the plasma in patients with vascular disease. Fibrinogen has been shown to inhibit myeloperoxi-

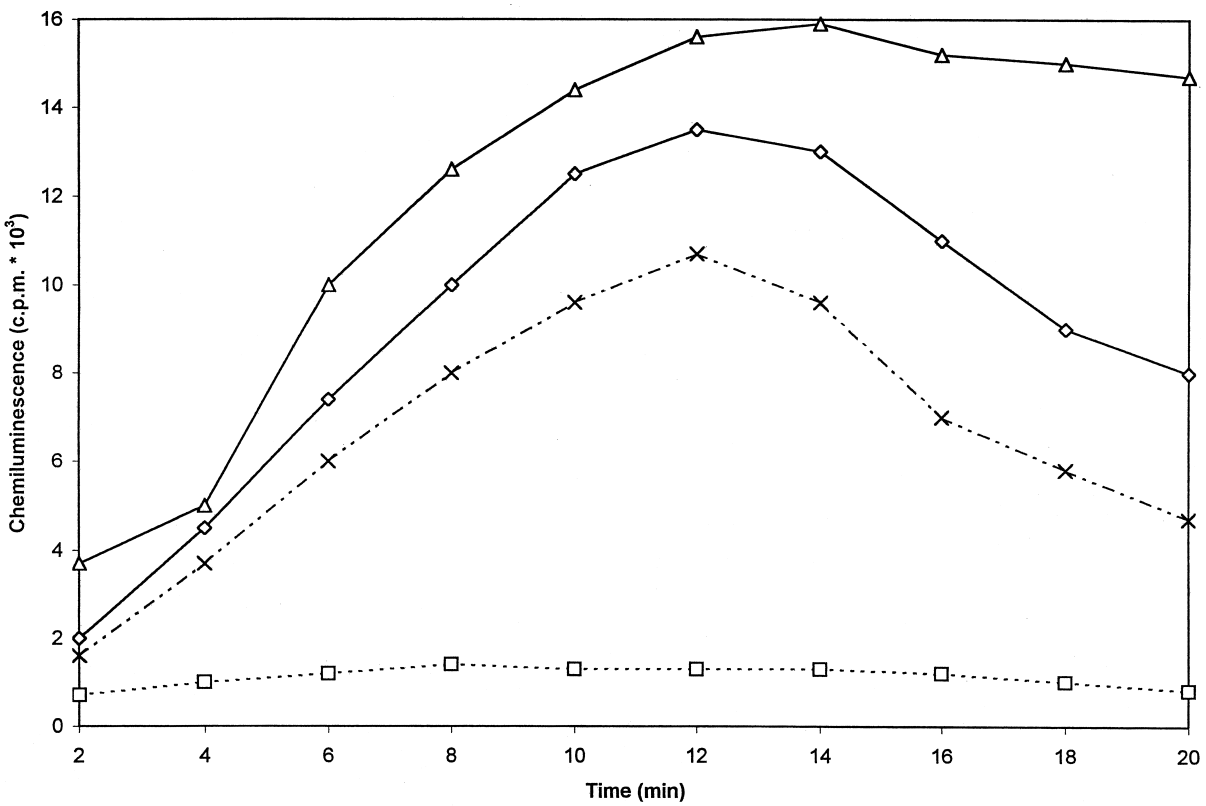

Fig. 3. The kinetics of CL emission produced by the opsonized zymosan induced PMN leukocytes activation without zymosan (□----□), with 1 mg of opsonized zymosan (◇----◇) and with 1 mg/ml (△——△) and 3 mg/ml (X----X) of additional purified fibrinogen.

dase-mediated oxidations especially at the upper physiological level [28,41,42]. The oxygen free radicals released from activated leukocytes seem to be the main source of oxidants. The inhibitory effect of FB on the CL produced during phagocytosis may have a physiological role. As already shown, FB as well as ceruloplasmin are synthesized at a higher rate as soon as an acute or extensive inflammatory condition occurs within the body [44].

The antioxidant function of FB increased when whole plasma was treated with a metal-catalyzed oxidation system [45] or irradiated with gamma rays [46]. This stimulatory effect of Fe^{2+} takes place in the first phase of peroxidation (1)

$$nFe^{2+} + nO_2^{\cdot -} \rightarrow nFe^{3+} + nO_2^{-} \quad (1)$$

as well as by enhancing decomposition of peroxides (LOOH)

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + OH^{-} + LO^{\cdot} \quad (2)$$

Karpel et al. [47] studied FB modification following the O_2 free radicals produced by a reaction between Cu^{2+} and ascorbate. In these experiments FB behaved as a true antioxidant, but these studies were aimed only at the coagulation process. If FB is acting as a sacrificing antioxidant, the consequence will lead to disturbances of the clotting process. Lee and Shacter [48] showed that FB is the most highly susceptible protein to oxidative modification compared to other plasma proteins and that oxidant-induced structural modifications are derived largely from amino acid oxidation. Both FB and albumin may act as antioxidants due to their amino acid backbone which serves as a "sink" or as sacrificial antioxidants according to their characterization by Halliwell et al. [28,29]. Albumin is known to be a powerful antioxidant [26,39,40], and its concentration in plasma exceeds that of FB. But the increase of acute phase proteins which occurs in inflammatory and neoplasm conditions involves antioxidant proteins with high molecular weights such as 230 KD for ceruloplasmin and 340 KD for FB. Thus, if supplementary protein antioxidants are required to counteract the high amounts of reactive oxygen species released by activated leukocytes, these high molecular weight antioxidant proteins are more suitable to prevent oxidation than albumin. An increase of albumin will disturb the osmotic equilibrium in the plasma. Fibrinogen, ceruloplasmin and possibly other acute phase proteins should be recognized as a supplementary antioxidant defense mechanism arising from inflammatory conditions.

Acknowledgments

We acknowledge John Mackie for the statistical analyses and the Wallace Genetic Foundation for financial support.

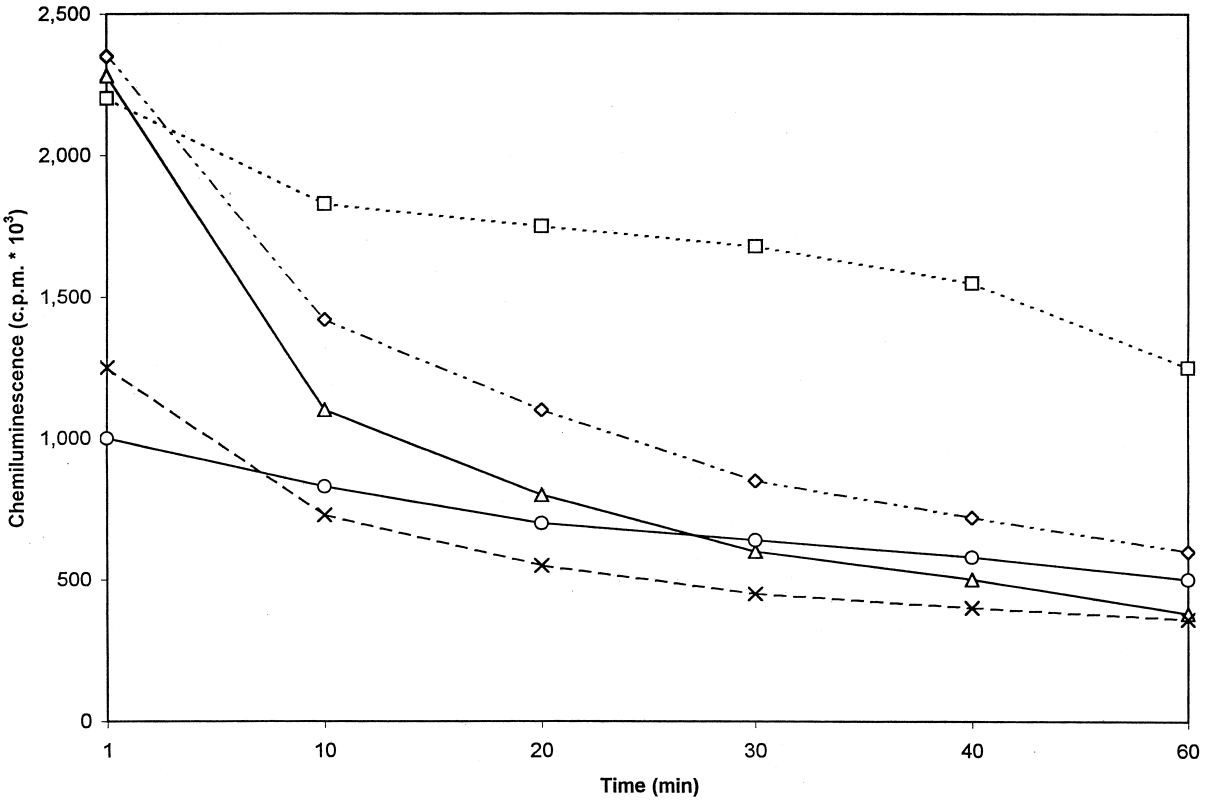

Fig. 4. The kinetics of CL emission in a cell-free system. The CL was produced by the reaction between luminol (100 μM) and H_2O_2 (10 mM). The CL emission of the generating system (□----□) was modified by the presence of 10 μM ascorbate (◇----◇), 8 μM (△——△) or 12 μM (X----X) fibrinogen, and 10 μM Trolox (◯——◯).

References

[1] L. Wilhelmsen, K. Svardsudd, K. Korsan-Bengtsen, B. Larrson, L. Welin, G. Tibblin. Fibrinogen as risk factor for stroke and myocardial infarction. N. Engl. J. Med. 311 (1984) 501–505.

[2] P. Levenson, J. Giral, J. Gariepy, A. Simon. Fibrinogen and silent atherosclerosis in subjects with cardiovascular risk factors. Arterioscler. Thromb. Vasc. Biol. 15 (1995) 1263–1268.

[3] S.L. Harley, J. Sturge, J.T. Powell. Regulation by fibrinogen and its products of intercellular adhesion molecule-1 expression in human saphenous vein endothelial cells. Arterioscler. Thromb. Vasc. Biol. 20 (2000) 652–658.

[4] T.H. Lam, L.J. Liu, E.D. Janus, C.P. Lau, A.J. Hedley. Fibrinogen, angina and coronary heart disease in a Chinese population. Atherosclerosis 149 (2000) 443–449.

[5] A.R. Folsom, K.K. Wu, M. Rasmussen, L.E. Chambless, N. Aleksic, F.J. Nieto. Determinants of population changes in fibrinogen and factor VII over 6 years: the Atherosclerosis Risk in Communities (ARIC) Study. Aterioscler. Thromb. Vasc. Biol. 20 (2000) 601–606.

[6] A. Tsutsumi, T. Theorell, J. Hallqvist, C. Reuterwall, U. de Faire. Association between job characteristics and plasma fibrinogen in normal working population: a cross sectional analysis in referents of the SHEEP study. Stockholm Heart Epidemiology Program. J. Epidemiol. Community Health 53 (1999) 348–354.

[7] G. Maresca, A. Di Blasio, R. Marchioli, G. Di Minno. Measuring plasma fibrinogen to predict stroke and myocardial infarction: an update. Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1368–1377.

[8] R.P. Tracy, E.G. Bovill, D. Yanez, B.M. Psaty, L.P. Fried, G. Heiss, M. Lee, J.F. Polak, P.J. Savage. Fibrinogen and factor VIII, but not factor VII, are associated with measures of subclinical cardiovascular disease in the elderly. Arterioscler. Thromb. Vasc. Biol. 15 (1995) 1269–1279.

[9] A. Bin, J.J. Fenoglio, R. Mesa-Tejada, B.J. Kudryk, K.L. Kaplan. Identification and distribution of fibrinogen, fibrin, and fibrin(ogen) degradation products in atherosclerosis. Use of monoclonal antibodies. Arteriosclerosis 9 (1989) 109–121.

[10] R. Lassila, S. Peltonen, M. Lepantalo, O. Saarinen, P. Kauhnanen. Severity of peripheral atherosclerosis is associated with fibrinogen and degradation of cross-linked fibrin. Arterioscler. Thromb. 13 (1993) 738–742.

[11] M. Navab, J.A. Berliner, A.D. Watson, S.Y. Hama, M.C. Territo, A.J. Lusis. The yin and yang of oxidation in the development of the fatty streak. Arterioscler. Thromb. Vasc. Biol. 16 (1996) 831–842.

[12] Heinecke, J.W. Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. Atherosclerosis 141 (1998) 1–15.

[13] I.B. Kovacs, M. Jahangiri, G.M. Rees, P. Gorog. Elevated plasma lipid hydroperoxides in patients with coronary artery disease. Am. Heart J. 134 (1997) 572–576.

[14] L. Mosca, M. Rubenfire, T. Tarshis, A. Tsai, T. Pearson. Clinical predictors of oxidized low-density lipoprotein in patients with coronary artery disease. Am. J. Cardiol. 80 (1997) 825–830.

[15] P. Holvoet, J.M. Stassen, J.V. Cleemput, D. Collen, J. Vanhaecke. Oxidized low density lipoproteins in patients with transplant-associated coronary artery disease. Arterioscler. Thromb. Vasc. Biol. 18 (1998) 100–107.

[16] E. Maggi, G. Finardi, M. Poli, P. Bollati, M. Filipponi, P.L. Stefano, G. Paolini, A. Grossi, P. Clot, E. Albano, et al. Specificity of autoantibodies against oxidized LDL as an additional marker for atherosclerotic risk. Coron. Artery Dis. 4 (1993) 1119–1122.

[17] C.P. Sparrow, T.W. Doebber, J. Olszewski, M.S. Wu, J. Ventre, K.A. Stevens, Y.S. Chao. Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant N,N′-diphenyl-phenylenediamine. J. Clin. Invest. 89 (1992) 1885–1891.
[18] N. Ide, B.H. Lau. Garlic compounds protect vascular endothelial cells from oxidized low density lipoprotein-induced injury. J. Pharm. Pharmacol. 49 (1997) 908–911.
[19] G. Murugesan, G.M. Chisolm, P.L. Fox. Oxidized low density lipoprotein inhibits the migration of aortic endothelial cells in vitro. J. Cell Biol. 120 (1993) 1011–1019.
[20] A. Negre-Salvayre, G. Fitoussi, V. Reaud, M.T. Pieraggi, J.C. Thiers, R. Salvayre. A delayed and sustained rise of cytosolic calcium is elicited by oxidized LDL in cultured bovine aortic endothelial cells. FEBS Lett. 299 (1992) 60–65.
[21] J. Nilsson. Lipoproteins and inflammation in atherosclerosis. Fibrinolysis & Proteolysis 11 (1) (1997) 129–132.
[22] H.N. Hodis, D.M. Kramsch, P. Avogaro, G. Bittolo-Bon, G. Cazzolato, J. Hwang, H. Peterson, A. Sevanian. Biochemical and cytotoxic characteristics of an in vivo circulating oxidized low density lipoprotein (LDL-). J. Lipid Res. 35 (1994) 669–677.
[23] D.D. Wayner, G.W. Burton, K.U. Ingold, L.R. Barclay, S.J. Locke. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim. Biophys. Acta 924 (1987) 408–419.
[24] J.M. Gutteridge. Antioxidant properties of the proteins ceruloplasmin, albumin and transferrin. A study of their activity in serum and synovial fluid from patients with rheumatoid arthritis. Biochim. Biophys. Acta 869 (1986) 119–127.
[25] A. Dobrian, R. Mora, M. Simionescu, N. Simionescu. In vitro formation of oxidatively-modified and reassembled human low-density lipoproteins antioxidant effect of albumin. Biochim. Biophys. Acta 1169 (1993) 12–24.
[26] S.A. Hulea, E. Wasowicz, F.A. Kummerow. Inhibition of metal-catalyzed oxidation of low-density lipoprotein by free and albumin-bound bilirubin. Biochim. Biophys. Acta 1259 (1995) 29–38.
[27] F.A. Kummerow, R.M. Olinescu, L. Fleischer, B. Handler, S.V. Shinkareva. The relationship of oxidized lipids to coronary artery stenosis. Atherosclerosis 149 (2000) 181–190.
[28] B. Halliwell. How to characterize a biological antioxidant. Free Radic. Res. Commun. 9 (1990) 1–32.
[29] B. Halliwell. Antioxidant characterization: methodology and mechanism. Biochem. Pharmacol. 49 (1995) 1341–1348.
[30] D. Lapenna, S. Degioda, A. Mazzetti, G. Ciofani, D. Feste, F. Cuccurullo. Aminophylline: could it act as an antioxidant in vivo? Europe J. Clin. Invest. 25 (1995) 464–470.
[31] I.M. Goldestein, H.B. Kaplan, H.S. Edelson, G. Weissman. Ceruloplasmin a scavenger of superoxide anion radicals. J. Biol. Chem. 254 (1979) 4040–4045.
[32] B. Tadolini, G. Pintus, G.G. Pinna, F. Bennardini, F. Franconi. Effects of taurine and hypotaurine on lipid peroxidation. Biochem. Biophys. Res. Commun. 213 (1995) 820–826.
[33] J.M.C. Gutteridge, S. Wilkins. Copper salt-dependent hydroxyl radical formation. Damage to proteins acting as antioxidants. Biochem. Biophys. Acta. 759 (1983) 38–41.
[34] J.M. Gaziano, A. Hatta, M. Flynn, E.J. Johnson, N.I. Wrinsky, P.M. Ridker, Ch.H. Hennekens, B. Frei. Supplementation with beta-carotene in vivo and in vitro does not inhibit low density lipoprotein oxidation. Atherosclerosis 112 (1995) 187–195.
[35] M. Marcat, L. Gerbant. An improvement of the Coomassie blue-dye binding method allowing an equal intensity to various proteins: application to cerebrospinal fluid. Clin. Chim. Acta 122 (1982) 93–101.
[36] M.A. Trush, M.E. Wilson, K. Van Dyke. In *Methods in Enzymology* (M. DeLucas, ed.). pp. 462–486, (1978), Academic Press, New York.
[37] R. Olinescu, S. Nita. A simple chemiluminescent system for the determination of total antioxidant capacity. Roum. J. Biophys. 2 (1992) 245–250.
[38] M.N. Hamers, D.H. Ross. Oxidative stress in human neutrophilic granulocytes. In *Oxidative stress* (H. Sies, ed.). pp. 351–75 (1985), Academic Press, London.
[39] B. Halliwell. Albumin—an important extracellular antioxidant? Biochem. Pharmacol. 37 (1988) 569–571.
[40] A. Bini, J.J. Fenoglio, R. Mesa-Tejada, B.J. Kudryk, K.L. Kaplan. Identification and distribution of fibrinogen, fibrin, and fibrin(ogen) degradation products in atherosclerosis. use of monoclonal antibodies. Arteriosclerosis 9 (1989) 109–121.
[41] R. Lassila, S. Peltonen, M. Lepantalo, O. Saarinen, P. Kauhnanen. Severity of peripheral atherosclerosis is associated with fibrinogen and degradation of cross-linked fibrin. Arterioscler. Thromb. 13 (1993) 1738–1742.
[42] R. Olinescu, S. Nita, D. Crocnan. On the significance of chemiluminescence induced by the blood polymorphonuclear leukocytes. Rev. Roum. Med. Int. 31 (1993) 109–112.
[43] A.A. Higazi, I.L. Barghouti, S.K. Ayesh, M. Mayer, Y. Matzner. Inhibition of neutrophil activation by fibrinogen. Inflammation 18 (1994) 525–535.
[44] E. Ernest. Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature. Annals Intern. Med. 118 (1993) 956–963.
[45] E. Shacter, J.A. Williams, R.L. Levine. Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. Free Rad. Biol. Med. 18 (1885) 815–821.
[46] E. Shacter, J.A. Williams, M. Lern, R. L. Levine. Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. Free Rad. Biol. Med. 17 (1994) 429–437.
[47] R. Karpel, G. Marx, M. Chevion. Free radical-induced fibrinogen coagulation: modulation of neofibe formation by concentration, pH and temperature. Israel J. Med. Sci. 27 (1991) 61–66.
[48] Y.J. Lee, E. Shacter. Role of carbohydrates in oxidative modification of fibrinogen and other plasma proteins. Archiv. Biochem. Biophys. 321 (1995) 175–181.