Oxidative Modification of Fibrinogen Is Associated With Altered Function and Structure in the Subacute Phase of Myocardial Infarction

Matteo Becatti, Rossella Marcucci, Giulia Brunghi, Niccolò Taddei, Daniele Bani, Anna Maria Gori, Betti Giusti, Gian Franco Gensini, Rosanna Abbate, Claudia Fiorillo

Objective—Among plasma proteins, fibrinogen represents a major target of oxidative modifications. In patients with post–acute myocardial infarction (6 months after the acute event), fibrinogen oxidation-induced carbonyls and fibrinogen function were estimated using in vitro and ex vivo approaches. Fibrinogen structural features and clot architecture were also explored.

Approach and Results—In 39 patients with post–acute myocardial infarction and 28 age-, sex-, and risk factor-matched controls, oxidative stress markers (in plasma and in purified fibrinogen fractions), thrombin-catalyzed fibrin polymerization, and plasmin-induced fibrin lysis were estimated. Circular dichroism spectra of purified fibrinogen extracts, electron microscopy, and differential interference contrast microscopy analyses of fibrin clots were also performed. Marked signs of oxidative stress in plasma ($P<0.01$ versus controls) and, correspondingly, an increased extent of fibrinogen carbonylation (3.5-fold over control values; $P<0.01$ versus controls) were observed in patients. Furthermore, fibrinogen fractions purified from patients exhibited significantly reduced clotting ability and decreased susceptibility to plasmin-induced lysis ($P<0.01$ versus controls). Alterations in fibrinogen secondary structure, as suggested by circular dichroism spectroscopy, and in fibrin clot architecture, as analyzed by electron and differential interference contrast microscopy, were also identified.

Conclusions—Here, we report for the first time that patients with post–acute myocardial infarction present with an overall imbalance in redox status and marked fibrinogen carbonylation associated with altered fibrinogen function, thus suggesting a role for carbonylation as a direct mechanism of fibrinogen function. The observed features occur along with modifications in protein structure and in clot architecture.

Key Words: fibrinogen • myocardial infarction

The imbalance between reactive oxygen species production and antioxidant defenses leads to the condition known as oxidative stress. Disturbances in the normal redox status of tissues can cause toxic effects through the production of peroxides and free radicals that damage all cellular components, including proteins, lipids, and DNA. Proteins are the main targets for reactive oxygen species that may alter every level of their structure from primary to quaternary, causing physical changes.1 Increased reactive oxygen species generation by vascular and inflammatory cells occurs in cardiovascular disease, and there is widespread evidence that oxidative injury contributes to vascular damage and cardiac dysfunction.2 Elevated levels of oxidative biomarkers, such as protein carbonyls (PC) and lipid peroxidation markers (thiobarbituric acid reactive substances, 8-epi-prostaglandin F), have been found in atherosclerotic lesions and in the circulation of patients with coronary artery disease (CAD).3–6 In plasma of patients with acute myocardial infarction (AMI) assayed within 24 to 96 hours after the acute event, an impaired fibrin lysis and an enhanced fibrinogen carbonylation has been recently observed.4,7

No evidence for a possible role of carbonylation on fibrinogen function in patients with post-AMI is available. Hence, for the first time, we investigated by an in vitro and an ex vivo approach, whether fibrinogen function—assessed by thrombin-catalyzed fibrin polymerization and fibrin susceptibility to plasmin-induced lysis—is associated with alterations in its oxidative status. Additional data on modifications in fibrinogen structure and in clot architecture are also reported.

Materials and Methods

Materials and Methods are available in the online-only Supplement.
Results

Subjects
Demographic and clinical characteristics of the population studied are summarized in Table 1.

Oxidative Stress Markers in Plasma and in Fibrinogen
As reported in Table 2, patient plasma displayed significantly higher total PC and thiobarbituric acid reactive substances levels, and lower total antioxidant capacity, than healthy controls ($P<0.05$ versus controls). Plasma total PC was positively and significantly correlated with fibrinogen PC ($P<0.05$; $R=0.555$; Figure 1A) and with thiobarbituric acid reactive substances levels ($P<0.05$; $R=0.444$; Figure 1B).

Purified fibrinogen from patients displayed significantly increased carboxylation (3.5-fold) in comparison with healthy controls ($P<0.01$; Figure 1C). PC amounts obtained in human, 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH)–challenged purified fibrinogen (SIGMA, Milan, Italy) were comparable with those observed in fibrinogen from patients with post-AMI and resulted significantly increased compared with untreated fibrinogen (Figure 3).

Circular Dichroism Spectra: Analysis of Secondary Structure
Secondary protein structure was analyzed by far-UV circular dichroism spectroscopy (Figure 2). In control subjects, the observed spectrum for fibrinogen suggested a typically $\alpha$-helical structure with minima at 208 and at 222 nm.

Table 1. Clinical Characteristics of Post–Acute Myocardial Infarction Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=39)</th>
<th>Controls (n=28)</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/Women (n, %)</td>
<td>27/12 (69.2)</td>
<td>20/8 (67.9)</td>
<td>0.999</td>
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<td>Age, y; median and interquartile range</td>
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<td>Hypertension (n, %)</td>
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<td>$\beta$-Blockers (n, %)</td>
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<td>&lt;0.001</td>
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</table>

ACE indicates angiotensin-converting enzyme; and CAD, coronary artery disease.

Table 2. Plasma Redox Status and Fibrinogen Carbonyl Content in Patients With Post-AMI and Control Subjects

<table>
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<tr>
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<th>Control Subjects</th>
<th>Patients With Post-AMI</th>
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<tbody>
<tr>
<td>TBARS, nmol/mL</td>
<td>11.2±3.3</td>
<td>30.5±13.6* (28.90)</td>
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<td>470.2±45.3</td>
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<td>Total PC plasma, nmol/mg</td>
<td>1.15±0.31</td>
<td>2.67±1.02* (2.73)</td>
</tr>
<tr>
<td>Fibrinogen carbonyl content, nmol/mg</td>
<td>0.19±0.05</td>
<td>0.66±0.32* (0.69)</td>
</tr>
</tbody>
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All data are represented as mean±SD. PC indicates protein carbonyls; post-AMI, post–acute myocardial infarction; TAC, total antioxidant capacity; and TBARS, thiobarbituric acid reactive substance.

Fibrinogen from patients with post-AMI displayed an altered circular dichroism spectrum consisting mainly of a decrease in the negative peak in the 215 to 225 nm region, therefore, suggesting a decrease in $\alpha$-helical content (Figure 2).

Fibrinogen Polymerization and Fibrin Formation
Representative curves of thrombin-catalyzed fibrinogen polymerization are shown in Figure 3. In an in vitro assay, increasing concentrations of AAPH affected thrombin-induced polymerization of pure fibrinogen (Figure 3A). In particular, in the presence of increasing AAPH concentrations, $V_{max}$ and Max absorbance progressively and significantly decreased, whereas lag time increased in a dose-dependent manner ($P<0.01$ versus controls).

To evaluate whether an antioxidant treatment could prevent the observed alterations, 0.05 mmol/L Trolox was added to the AAPH incubation reactions and thrombin-catalyzed polymerization of fibrinogen was performed. As Figure 3A reports, the simultaneous incubation of AAPH with Trolox was able to prevent the observed changes.

Similarly, in patients, the ability of fibrinogen to undergo clotting was diminished: significant differences in lag time, $V_{max}$, and Max absorbance versus controls were found ($P<0.01$; Figure 3B). In Figure 3C, the correlation analysis between fibrinogen carbonyl content and Max absorbance was reported: the relationship seems inverse and significant ($P<0.05$ $R=0.419$).

Transmission Electron Micrographs and Differential Interference Contrast Microscopy
Using electron microscopy, we found that fibrin filaments from patients and controls examined preplasmin digestion displayed differences in size and morphology: in particular, control fibrin filaments seemed as bundles of densely packed, longitudinally arranged fibrin molecules (Figure 4A, top), whereas post-AMI fibrin filaments consisted of thinner fibrin fibers (mean diameter, 120±13.2 nm versus control, 151±13.6 nm; Figure 4B). After plasmin digestion, samples from control subjects showed a complete disarrangement of fibrin filaments, with short fibrin monomers forming a loose 3-dimensional network (Figure 4A, lower left). Samples from patients with post-AMI after plasmin digestion still showed a filamentous pattern although with slightly decreased average fiber size (mean diameter, 89±10.9 nm versus control 40±3.7 nm).
nm; Figure 4B) and evident fibrin monomers (Figure 4A, lower right).

In Figure 4A, fibrin samples obtained from patients with post-AMI and controls analyzed by differential interference contrast microscopy are shown. A tight fibrin network is still evident after plasmin-induced lysis in fibrin obtained from patients with post-AMI when compared with that obtained from controls.

**Fibrin Susceptibility to Plasmin-Induced Lysis**

Fibrin β-chain degradation at 0, 3, and 6 hours after plasmin digestion in vitro experiments consisting of human purified fibrinogen treated with AAPH (in the absence or presence of Trolox) and quantification of residual β-chain intensity after 6 hours plasmin digestion is reported in Figure 5A. Fibrin clots obtained with pure fibrinogen after incubation with increasing AAPH concentrations showed reduced susceptibility to plasmin-induced lysis, at each considered time of plasmin digestion. To evaluate whether an antioxidant treatment could prevent the observed alterations, 0.05 mmol/L Trolox was added to AAPH incubation reactions and fibrin susceptibility to plasmin-induced lysis was then assessed. As shown in Figure 5A, the simultaneous incubation of AAPH and Trolox was able to prevent the observed changes in fibrin digestion by plasmin.

Fibrin β-chain degradation after 0, 3, and 6 hours of plasmin digestion in patients with post-AMI and controls is reported in Figure 5B. In patients with post-AMI, the relative band intensity at each considered time of plasmin digestion was significantly higher with respect to controls.

In patients, the correlation between fibrinogen carbonyl content and the relative fibrin β-chain intensity after 6 hours of plasmin digestion was shown to be positive and significant (P<0.05; Figure 5C). In patients with post-AMI, fibrin β-chain intensity after 6 hours of plasmin digestion was also significantly correlated with plasma PC (P<0.01; R=0.540) and thiobarbituric acid reactive substances levels (P<0.01; R=0.616, data not shown).

**Discussion**

The results of the present study show, for the first time, that in patients with post-AMI (1) a systemic redox imbalance is coupled with an increased level of fibrinogen carbonylation; (2) changes in the secondary protein structure of fibrinogen are present; (3) the increased extent of fibrinogen carbonylation is associated with altered polymerization and susceptibility to plasmin-induced lysis; and (4) marked modifications in the global clot architecture are evident.

Atherosclerosis is characterized by lipid and protein oxidation in the vascular wall that contributes to important clinical manifestations of CAD represented by plaque disruption and endothelial dysfunction.2,8

Increased levels of oxidative biomarkers and decreased antioxidant activity in the circulating blood of patients with CAD have been reported,9,10 whereas raised PC values have also been found in patients with myocardial infarction, providing further evidence for enhanced oxidative stress in these subjects.11
In the present study, both plasma PC and fibrinogen PC were markedly and significantly increased in patients with post-AMI when compared with control subjects and were significantly correlated. In this context, previous reports indicate that fibrinogen is more susceptible to oxidation than most other plasma proteins; in particular, fibrinogen is 20× more susceptible to oxidation than albumin, as also reported in patients with MI, where it was found that total plasma carbonyls were formed preferentially on fibrinogen.

When we investigated, using far-UV circular dichroism spectroscopy, whether the alterations observed in fibrinogen functions could be related to secondary structure modifications, we found a shift in the mean residual ellipticity and a reduction in α-helical content in patients with post-AMI in comparison with controls, suggesting a change in secondary structure content. Taking into consideration that secondary structure of proteins plays a major role in determining their functionality in specific physiological processes, this preliminary result needs further investigation and leads to speculation that carbonylation of proteins plays a major role in determining their functionality.

In our study, the relationship between oxidized fibrinogen and fibrinogen function has been explored by measuring the clotting ability of purified fibrinogen using an in vitro thrombin-catalyzed polymerization assay. In this system, the magnitude of the turbidity increase relates to the architecture of the formed clot; the altered maximum absorbance of fibrin polymerization reflects the formation of thinner and more compact fibers.

Interestingly, the functional alterations that we observed when fibrinogen was subjected to oxidation could easily be reversed by simultaneous treatment with a water soluble analogue of α-tocopherol (Trolox), emphasizing the role of oxidative modification of fibrinogen on its function.

In patients with post-AMI, we observed a slower rate of thrombin-catalyzed fibrinogen polymerization; this alteration in fibrinogen function was found to be directly and significantly correlated to fibrinogen carbonyl content, suggesting an influence of carbonylation on thrombin-induced polymerization of fibrinogen.

The introduction of carbonyl groups into proteins can be triggered by different reactive oxygen species or secondary by-products of oxidative stress and can arise at different sites and by different mechanisms. Carbonylation can result in several different protein modifications, which may specifically affect the biological activity of proteins. Among the functional effects that have been described, modified binding activities, enzyme inactivation, and altered susceptibility to proteolytic degradation are the most represented. It has been shown that the introduction of carbonyl derivatives (aldehydes and ketones) may alter the conformation of the polypeptide chain, thus determining functional modification of proteins. The 2 amino acids that are perhaps the most prone to oxidative attack are cysteine and methionine, both of which contain susceptible sulfur atoms. Other amino acyl moieties, especially lysine, arginine, proline, and threonine, incur formation of carbonyl groups on the side chains. The presence of oxidizable amino acids (Pro and Arg) in the cleavage site of thrombin, or the cleavage of peptide bonds by oxidation of glutamyl residues, may explain the altered thrombin-induced fibrin polymerization observed in patients with post-AMI.

In a recent in-depth study of the thrombin cleavage site, the authors show that mutating both Pro and Arg results in a 200- to 400-fold drop in cleavage, which highlights the importance of these 2 positions for maximal substrate cleavage.

In our experimental model, carbonylation of purified human fibrinogen was responsible for an oxidant-dependent decrease in thrombin-induced clot formation likely determined by a covalent protein modification. In line with this, the observed alteration in clotting ability of purified human fibrinogen was completely prevented when it was incubated with both the oxidant generator AAPH and the antioxidant Trolox.

Our findings showing alteration of fibrinogen function strengthen other reports, showing that fibrinogen oxidation impairs the capacity of isolated fibrinogen to form a fibrin clot under the effect of thrombin; these results seem to contrast with those reported by Paton et al, obtained in 12 patients with MI, which were performed in the acute phase of myocardial infarction (ie, within 24–96 hours of the acute event). This discrepancy could be because of the different clinical setting: the

**Table 3. In vitro Assay: Protein Carbonyl Content in AAPH-Treated Pure Fibrinogen**

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>0.01 mmol/L AAPH</th>
<th>0.05 mmol/L AAPH</th>
<th>0.1 mmol/L AAPH</th>
<th>1 mmol/L AAPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure fibrinogen carboxyl content, nmol/mg</td>
<td>0.1±0.02</td>
<td>0.41±0.12*</td>
<td>1.22±0.22*</td>
<td>2.09±0.41*</td>
<td>9.19±1.32*</td>
</tr>
</tbody>
</table>

All data are represented as mean±SD. AAPH indicates 2′-azobis(2-aminopropane) dihydrochloride.

*Statistically significant difference vs untreated at the P<0.05 level, as assessed using the ANOVA-Bonferroni test.
acute phase is characterized by an inflammatory and hypercoagulable state in which molecular alteration of fibrinogen because of fibrinopeptide A release was demonstrated. Accordingly, a clear difference related to the clinical phase of CAD was documented by Undas et al, who reported significant differences in fibrinogen/fibrin alterations between patients with acute coronary syndrome and patients with stable angina. To establish another important aspect of fibrinogen function in relation to carbonylation, we estimated fibrin resistance to plasmin-induced lysis. We confirmed that in patients with post-AMI, fibrin is resistant to lysis and that its degradation is significantly lowered with respect to healthy controls. Our results agree with previous investigations, demonstrating that patients with premature CAD produce abnormal plasma fibrin clots ex vivo that are resistant to fibrinolysis. Interestingly, one of our main results is a strong and positive correlation between fibrinogen PC and residual β-chain intensity after plasmin-induced lysis. We confirmed that in patients with post-AMI, fibrin is resistant to lysis and that its degradation is significantly lowered with respect to healthy controls. Our results agree with previous investigations, demonstrating that patients with premature CAD produce abnormal plasma fibrin clots ex vivo that are resistant to fibrinolysis. Interestingly, one of our main results is a strong and positive correlation between fibrinogen PC and residual β-chain intensity after plasmin-induced lysis. A similar correlation was found when correlating residual β-chain intensity after plasmin-induced lysis with plasma PC. The reported findings are consistent with the observation that F2-isoprostanes, which are stable markers of oxidative stress, are associated with reduced clot permeability and fibrinolysis in patients with cardiovascular.
Moving from the finding of altered thrombin-induced fibrin polymerization, possibly characterized by the presence of impaired lateral association of protofibrils and by alteration in fiber size resulting from fibrinogen carbonylation, we then performed an in-detail analysis of clot structure by electron and differential interference contrast microscopy. Our main finding relates to the presence, in post-AMI fibrin, of a tight fibrin network composed of filaments with slightly decreased average fiber size that are resistant to plasmin-induced lysis when compared with control subjects. Actually, clot structure is determined by several factors. In most purified systems, as fiber size increases, pore size increases too. Fibrin networks composed of thin fiber strands have small pores and are more rigid and less permeable. In contrast, clots formed by thick fiber strands have large liquid spaces, which imply higher permeability and accelerated fibrinolysis, likely because of a more efficient transport of fibrinolytic agents through a fibrin clot. Clots composed of thin fibers and small pores are more thrombogenic and are associated with CAD. The mechanisms underlying formation of these abnormal fibrin clots have not yet been elucidated but could be related to post-translational modifications of fibrinogen. The data presented here suggest a causative link between fibrinogen carbonylation and the structural alterations observed in patients with post-AMI. An interesting aspect that must be taken into consideration is that all patients enrolled in the study were given aspirin as a secondary prevention treatment strategy, which could also have had an effect on clot architecture. Fibrinogen, in fact, is rich in lysine residues, which are the main target of acetylation by aspirin, treatment with which may disturb charge distribution on the protein surface. This action, however, has been shown to not affect the rate of fibrinogen gelation by thrombin.

In conclusion, our in vitro and ex vivo findings show that in patients with post-AMI, an overall imbalance in redox status and marked fibrinogen carbonylation are associated with altered clotting activity and susceptibility of plasmin to lysis. These features, observed far from the acute event, are accompanied by an alteration in protein structure and clot architecture.

Sources of Funding

This study was funded by the Italian Ministry of Health 2009, Research Project title: “Antiplatelet therapy tailored by platelet function and pharmacogenetic profile: toward an appropriate use of the new antiplatelet agents” and by the University of Florence (Fondi di Ateneo), Italy.

Disclosures

None.

References


Fibrinogen represents an important target of oxidative modifications. In patients with post–myocardial infarction (6 months after the acute event), an overall imbalance in plasma redox status and marked fibrinogen carbonylation were associated with impaired clotting activity and reduced plasmin susceptibility to lysis. Fibrinogen protein secondary structure and clot architecture were also markedly altered. The features described here, observed far from the acute event, provide new insights into the mechanisms that control fibrin structure and function. These new findings might allow the development of pharmaceutical strategies to modulate fibrin structure in vivo and thereby might be useful for the primary and secondary prevention of coronary artery disease.
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<tr>
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Table 2: Plasma redox status and fibrinogen carbonyl content in post-AMI patients and control subjects.

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<th>CONTROL SUBJECTS</th>
<th>POST-AMI PATIENTS</th>
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<tr>
<td>TBARS (nmol/ml)</td>
<td>11.2±3.3</td>
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All data are represented as means±SD. * Statistically significant difference vs Control at the p<0.05 level, as assessed using the ANOVA-Bonferroni test.
### Table 3: *In vitro* assay: Protein carbonyl content in AAPH-treated pure fibrinogen

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<td>0.1 ±0.02</td>
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OXIDATIVE MODIFICATION OF FIBRINOGEN IS ASSOCIATED WITH ALTERED FUNCTION AND STRUCTURE IN THE SUBACUTE PHASE OF MYOCARDIAL INFARCTION

MATTEO BECATTI¹, ROSSELLA MARCUCCI², GIULIA BRUSCHI¹, NICCOLÒ TADDEI¹, DANIELE BANI³, ANNA MARIA GORI², BETTI GIUSTI², GIAN FRANCO GENSINI²,³, ROSANNA ABBATE² AND CLAUDIA FIORILLO¹*

¹ Department of Experimental and Clinical Biomedical Sciences, ² Department of Clinical and Experimental Medicine, ³ Department of Clinical and Experimental Medicine, Research Unit of Histology and Embriology University of Florence, Florence, Italy; ⁴ Don Carlo Gnocchi Foundation, Florence, Italy.

Materials and Methods

Bovine thrombin, human plasmin and fibrinogen from human plasma were purchased from Sigma-Aldrich (Milan, Italy); Criterion XT Precast Gels 4-12% Bis-Tris were from Bio-Rad (Milan, Italy). All other chemicals were of the highest purity available.

Study population

The study sample included 39 post-AMI patients and 28 healthy age- and sex-matched subjects. The characteristics of patients and controls are given in table 1.

Patients with post-myocardial infarction who underwent PCI within the previous 6 months were considered eligible for the study. The only exclusion criteria were in-hospital death and anticipated non-adherence to dual antiplatelet treatment for at least 6 months. Informed written consent was obtained from all patients and the study was approved by the local Ethical Review Board.

Percutaneous coronary intervention and antiplatelet management

All interventions were performed according to current standard guidelines, and the type of stent implanted and the use of IIb/IIIa inhibitors were at the discretion of the operator. All patients received one clopidogrel loading dose of 600 mg followed by a daily dose of 75 mg. All patients received unfractioned heparin 70 IU/Kg during the procedure and acetylsalicylic acid i.v. 500 mg, followed by an orally administered daily dose of 100-325 mg according to the current guidelines for PCI.

Sample collection

Blood samples were collected 6 months after myocardial infarction in Vacutainer tubes containing 0.109 mol/L buffered trisodium citrate (1:10) or EDTA (0.17 mol/L). After centrifugation (1500xg for 15 minutes at 4 ºC), aliquots of plasma were used for experiments or stored at -80 ºC for further analysis. Another aliquot of sodium citrate plasma was used for fibrinogen purification.

Fibrinogen purification

Fibrinogen was purified using the previously described ethanol precipitation method.¹
After the purification procedure, fibrinogen concentration was determined by ultraviolet spectroscopy at a wavelength of 280 nm, assuming an extinction coefficient of 1.51 \( \text{(mg/ml)}^{-1} \). The yield of purified fibrinogen was not statistically different between patients and controls (9.9±1.9 vs 9.1±1.7, mg/10 ml of plasma, respectively). The purity of the fibrinogen preparations (from 10 ml of citrated plasma) was assessed by densitometry of Coomassie-stained polyacrylamide gels after electrophoresis under reducing conditions. In our purification procedures, the amount of fibrinogen, expressed as a percentage of total protein content, yielded 96.7±1.1% of total protein content in controls and 95.3±0.9% in post-AMI patients. No significant statistical difference was observed in the purification yield between controls and patients.

**Protein Concentration assay**

Protein concentration in the samples was determined using the Bradford assay.\(^2\) A standard curve of bovine serum albumin (0–15 \( \mu \text{g protein/200 } \mu\text{l volume} \)) was used.

**Protein Carbonyl (PC) determination**

Oxidative modification on plasma proteins and on purified fibrinogen fractions (untreated and treated with AAPH) was assessed based on carbonyl content using 2,4 dinitrophenylhydrazine (DNPH), as previously reported.\(^3\)

DNPH reacts with PC, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically. Briefly, plasma (100\(\mu\)l), after incubation with DNPH (400\(\mu\)l), was precipitated with Trichloracetic acid (TCA) and the pellet washed several times with a 1:1 mixture of ethanol/ethyl acetate. Finally, the pellet was resuspended in 500\(\mu\)l of guanidine hydrochloride and measured at 370 nm. PC content was calculated by using a molar extinction coefficient of 22000 \( \text{M}^{-1} \text{cm}^{-1} \). The results, expressed in terms of nmol/ml of PC, were then normalized for protein concentration.

**TBARS (Thiobarbituric Acid Reactive Substances) estimation**

Plasma TBARS levels were measured using a TBARS assay kit (OXI-TEK, ENZO, USA) in accordance with the manufacturer's instructions. Briefly, the adduct generated by reacting malondialdehyde with Thiobarbituric acid after 1h at 95 °C was measured spectrofluorimetrically, with excitation at 530 nm and emission at 550 nm. TBARS were expressed in terms of malondialdehyde equivalent (nmol/ml) and then normalized for protein concentration.

**Total antioxidant capacity (TAC) assay**

The ORAC method (Oxygen Radical Absorbance Capacity), based on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds, like 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH), was performed as previously described.\(^4\) Briefly, a fluorescein solution (6 \( \text{nM} \)) prepared daily from a 4 \( \mu \text{M} \) stock in 75 mM sodium phosphate buffer
(pH 7.4), was used. Trolox (250 µM final concentration) was used as a standard. 70 µl of sample with 100 µl of fluorescein were pre-incubated for 30 min at 37 °C in each well, before rapidly adding AAPH solution (19mM final concentration). Fluorescence was measured with excitation at 485 nm and emission at 537 nm in a Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher Scientific Inc. MA, USA). Results were expressed as Trolox Equivalents (µM) and then normalized for protein concentration.

Circular Dichroism (CD) spectra of purified fibrinogen extracts

CD Spectra were recorded at 25 °C in 0.2 cm quartz cells from 250 to 195 nm (far UV), using a protein concentration of 1 mg/ml. Samples were filtered through 0.22 µM filters and three spectra recorded for each sample. Molar ellipticity values [θ] were calculated according to the equation: [θ] (deg·cm² dmol⁻¹) = [θ (MRW)]/[10(l)(c)], where θ is the displacement from the baseline value X to the full range in degrees; MRW is the mean residue weight of the amino acids; (l) is the path length of the cell (cm); and (c) is protein concentration (g/ml). ⁵

Thrombin-catalyzed fibrinogen and polymerization assays

For functional analysis, purified fibrinogen fractions stored at −80 °C and not previously thawed were used. Fibrin polymerization was monitored at 595 nm in a 96-well microtiter plate reader (model 550, Bio-Rad Milan, Italy) at 25 °C, as previously described.⁶ Prior to the polymerization assay, control and patient fibrinogen samples were extensively dialyzed against 100 mM Tris/HCl buffer, pH 7.4, and diluted to a final concentration of 1 mg/ml. To each reaction (in triplicate), 240 µl of fibrinogen (1 mg/ml) in 100 mM Tris/HCl, 5 mM CaCl₂, pH 7.4 was added. The polymerization reaction was started by adding 60 µl thrombin (at a final concentration of 0.25 U/ml). Absorbance was monitored for 90 min at 25 °C.

Absorbance curves were characterized using the following parameters: 1) the maximum slope (Vmax), calculated as the slope of the steepest part of the polymerization curve (using 10 time points), which represents the rate of lateral protofibril association; 2) the lag phase, measured as the time elapsed until an increase in absorbance was seen, which reflects the time to the start of lateral fibril aggregation; 3) maximum absorbance (Max Abs) of the growing clot, recorded 60 min after polymerization was initiated, which reflects an average fibrin fiber size and the number of protofibrils per fiber. ⁷

In another set of experiments, increasing concentrations (0.01mM -1mM) of AAPH were incubated with 2 mg of purified human fibrinogen (Sigma, Milan, Italy) dissolved in 1 ml phosphate buffered saline pH 7.4. Samples were incubated at 37° C for 24 h. To eliminate any AAPH residues in the samples, fibrinogen was recovered and dialyzed against PBS before the assay was conducted.
To evaluate the potential preventive effect of an antioxidant on the afore-mentioned AAPH-induced oxidation reaction, 2 mg of purified human fibrinogen (Sigma, Milan, Italy) dissolved in 1 ml phosphate buffered saline pH 7.4 was incubated with 0.05 mM AAPH in the presence of 0.05 mM Trolox at 37° C for 24 h. To eliminate any AAPH residues in the samples, fibrinogen was then recovered and dialyzed against PBS before the assay was carried out. The obtained samples were used for thrombin-catalyzed fibrinogen assays, as described above.

Transmission Electron Microscopy

Fibrin clots were prepared by incubating bovine thrombin (12 units/mL final concentration) with fibrinogen (2 mg/mL final concentration) in 20 µL of 100 mM Tris/HCl, 5 mM CaCl₂, pH 7.4 for 1 h at 25 °C. Plasmin was then added (5 µL of 100 µg/mL) and the fibrin clots digested over a period of 6 h at 37 °C, as previously described.¹ For Transmission Electron Microscopy fibrin samples were pelleted by centrifugation (800xg) in Eppendorf tubes, fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 24 h, post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 1 h, dehydrated in graded acetone and embedded in Epon 812 epoxy resin (Fluka, Buchs, CH). Ultrathin sections were stained with uranyl acetate and alkaline bismuth subnitrate and examined under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV. The mean diameter of fibrin filaments from the different experimental groups was measured by computer-aided morphometry on 50,000 x electron micrographs, using the freely available ImageJ 1.33 image analysis program (http://rsb.info.nih.gov/ij). For each sample, 50 filaments or more were measured and the values averaged.

Determination of fibrin structure based on label-free differential interference contrast (DIC) microscopy.

Fibrin clots prepared as described above were analyzed by DIC microscopy. This is a label-free microscopy technique with a high sensitivity to thin cellular material, even when it is located within thick tissue.⁸ DIC microscopy is superb for observing transparent objects and very thin filaments or sharp interfaces, which produce good contrast even when their diameter falls below the resolution limit of the optical system. DIC microscopy causes one side of an object to appear bright while the other side appears darker. This shadow effect gives a pseudo-three-dimensional appearance to the specimen, at excellent resolution.

Purified fibrinogen fractions stored at −80 °C and not previously thawed were used. Before polymerization, control and patient fibrinogen samples were extensively dialyzed against 100 mM Tris/HCl buffer, pH 7.4, and diluted to a final concentration of 1 mg/ml. To each reaction (in duplicate) 240 µl of fibrinogen (1 mg/ml) in 100 mM Tris/HCl, 5 mM CaCl₂, pH 7.4 was seeded on glass coverslips. The polymerization reaction was started by adding 60 µl of thrombin (at a final concentration of 0.25 U/ml) at 25 °C. After 90 min the sample was analyzed using a Leica TCS

¹ Reference suppressed for clarity.
SP5 microscope (Mannheim, Germany), using DIC. The observations for each examined sample were obtained using a Leica Plan Apo 63× oil immersion objective.

**Fibrin digestion with plasmin and electrophoretic analysis of plasmin digests**

Fibrin clots were prepared in microcentrifuge tubes by incubating bovine thrombin (12 units/mL final concentration) with fibrinogen (2 mg/mL final concentration) in 20 μL of 100 mM Tris/HCl, 5 mM CaCl₂, pH 7.4, for 1 h at 25 °C. Plasmin was then added (5 μL of 100 μg/mL), and the fibrin clots were digested over a period of 6 h at 37 °C, as previously described.² The digestion reaction was terminated by adding 10 μL of lithium dodecyl sulfate (LDS) gel electrophoresis sample buffer. The same lot of thrombin and of plasmin were used for all experiments. Samples were heated at 70 °C for 10 min under reducing conditions (50 mM dithiothreitol). Then, aliquots from each digest (equivalent to 10 μg of fibrin) were loaded onto 4–12% Bis-Tris gels. After electrophoresis, gels were stained with Coomassie blue. Band intensities of stained gels were quantified by densitometry using the Chemi-Doc system and Quantity-One software (Bio-Rad, Milan, Italy). Data were expressed as the ratio between the densitometric reading of the purified protein at a given digestion time and that of the undigested protein (time 0 for incubation with plasmin).

In another set of experiments, increasing concentrations (0.01mM -0.05mM) of AAPH dissolved in PBS, or PBS alone, were incubated with 2 mg of human purified fibrinogen (Sigma, Milan, Italy) dissolved in 1 ml phosphate buffered saline, pH 7.4. Samples were placed in a 37° C incubator for 24 hours. To eliminate any AAPH residues in the samples, fibrinogen was recovered and dialyzed against PBS prior to the assay. Fibrin clots were then prepared as described above, plasmin was added and digestion conducted over a period of 6 h at 37 °C.

To evaluate the potential preventive effect of an antioxidant on the above reaction, 2 mg of human purified fibrinogen (Sigma, Milan, Italy) dissolved in 1 ml phosphate buffered saline, pH 7.4, was incubated with 0.05mM AAPH in the presence of 0.05mM Trolox at 37° C for 24 h. To eliminate any AAPH residues in the samples, fibrinogen was recovered and then dialyzed against PBS prior to the assay. The obtained samples were clotted, digested with plasmin and subjected to electrophoresis as described above.

**Statistical Analysis**

All experiments were performed in triplicate. For descriptive aspects, owing to the symmetry of the distribution, data are summarized as means±SD. The statistical significance of the differences observed in the various groups considered in this study was assessed using the ANOVA-Bonferroni test. p<0.05 was accepted as statistically significant. Correlation analysis was performed using the Pearson’s test. All statistical operations data were processed using the program GraphPad Prism 5.
REFERENCES


