Pro-thrombotic State Induced by Post-translational Modification of Fibrinogen by Reactive Nitrogen Species*

Received for publication, June 10, 2003, and in revised form, December 12, 2003 Published, JBC Papers in Press, December 17, 2003, DOI 10.1074/jbc.M306101200

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Formation of nitric oxide-derived oxidants has been linked to development of atherosclerosis and associated thrombotic complications. Although systemic levels of protein nitrotyrosine predict risk for coronary artery disease, neither specific proteins targeted for modification nor functional consequences that might contribute to disease pathogenesis have been defined. Here we report a selective increase in circulating levels of nitrated fibrinogen in patients with coronary artery disease. Exposure of fibrinogen to nitrating oxidants, including those produced by the myeloperoxidase-hydrogen peroxide-nitrate system, significantly accelerates clot formation and factor XIII cross-linking, whereas exposure of fibrinogen to non-nitrating oxidants decelerates clot formation. Clots formed with fibrinogen exposed to nitrating oxidants are composed of large bundles made from twisted thin fibrin fibers with increased permeation and a decrease in storage modulus G’ value, suggesting that these clots could be easily deformed by mechanical stresses. In contrast, clots formed with fibrinogen exposed to non-nitrating oxidants showed decreased permeation with normal architecture. Fibrinogen modified by exposure to physiologic nitration systems demonstrated no difference in the rate of plasmin-induced clot lysis, platelet aggregation, or binding. Thus, increased levels of fibrinogen nitration may lead to a pro-thrombotic state via acceleration in formation of fibrin clots. The present results may account, in part, for the association between nitrative stress and risk for coronary artery disease.

Epidemiological studies have indicated that increased levels of circulating fibrinogen is an independent predictor of coronary heart disease and in some cases of premature death from cardiovascular disease, although a causative relationship between high levels of fibrinogen and cardiovascular disease has not been firmly established (1–4). Fibrinogen is a multifunctional protein essential for hemostasis. It is a 340-kDa glycoprotein, consisting of three non-identical peptide chains α, β, and γ, which are linked together by 29 disulfide bonds (5). During coagulation, the soluble fibrinogen is converted to insoluble fibrin polymers. The process is initiated by thrombin, a serine protease, which catalyzes the cleavage first of two fibrinopeptides from the amino termini of the αα chains and then two fibrinopeptides from the amino termini ββ chains. Upon release of the fibrinopeptides, the remaining fibrin monomers aggregate spontaneously to form ordered fibrin polymers (5). The clot is stabilized by the formation of covalent bonds introduced by the action of a transglutaminase, factor XIII (6). Under physiological conditions, fibrinolysis is dependent on the binding of circulating plasminogen and tissue-type plasminogen activator (tPA)† to fibrin clots. Urokinase and tPA convert plasminogen to the active protease plasmin, which then cleaves fibrin polymers to soluble fragments completing the coagulation and clot resolution cycle.

A major cause of vascular injury leading to the development of atherosclerosis is oxidative stress (7–9). Proteins are major targets for reactive species, and nitration of tyrosine residues is a selective protein modification induced by reactive nitrogen species in human disorders as well as animal and cellular models of disease (10, 11). Nitrated proteins have been detected in atherosclerotic lesions (12–16), and recent studies (17) show that the levels of nitrated plasma proteins independently predict risk for coronary artery disease. In this study we identified and quantified nitrated fibrinogen in the plasma of patients with documented coronary artery disease. In a series of experiments the effects of nitration on the kinetics of fibrin formation, factor XIII cross-linking, fibrin architecture and rheology, platelet aggregation and binding, and lysis by plasmin were determined and contrasted with oxidized and control fibrinogen.

MATERIALS AND METHODS

Human Studies—Sequential patients presenting to the Cardiology Section of the Cleveland Clinic Foundation or responding to local advertisements were enrolled. To be classified as having coronary artery disease, although a causative relationship between high levels of fibrinogen and cardiovascular disease has not been firmly established (1–4). Fibrinogen is a multifunctional protein essential for hemostasis. It is a 340-kDa glycoprotein, consisting of three non-identical peptide chains α, β, and γ, which are linked together by 29 disulfide bonds (5). During coagulation, the soluble fibrinogen is converted to insoluble fibrin polymers. The process is initiated by thrombin, a serine protease, which catalyzes the cleavage first of two fibrinopeptides from the amino termini of the αα chains and then two fibrinopeptides from the amino termini ββ chains. Upon release of the fibrinopeptides, the remaining fibrin monomers aggregate spontaneously to form ordered fibrin polymers (5). The clot is stabilized by the formation of covalent bonds introduced by the action of a transglutaminase, factor XIII (6). Under physiological conditions, fibrinolysis is dependent on the binding of circulating plasminogen and tissue-type plasminogen activator (tPA)† to fibrin clots. Urokinase and tPA convert plasminogen to the active protease plasmin, which then cleaves fibrin polymers to soluble fragments completing the coagulation and clot resolution cycle.

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† The abbreviations used are: tPA, tissue-type plasminogen activator; HPLC, high pressure liquid chromatography; MPO, myeloperoxidase; HOCl, hypochlorous acid; ME, microemboli; ARDS, acute respiratory distress syndrome; ANOVA, analysis of variance.

4 This work was supported by National Institutes of Health Grants P50-HL70128 (to J. W. W. and H. I.), HL70621, and HL62526 and GCRC Grant M01 RR018390 (to S. L. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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After incubation with the different chemical systems, the samples were applied to a PD-10 desalting column and were eluted with TBS (50 mM Tris base, 150 mM sodium chloride, pH 7.4). Following the different exposures, the nitration of fibrinogen was confirmed by Western blotting with anti-nitrotyrosine antibodies and by liquid chromatography/electrospray ionization/mass spectrometry analysis (Table I), whereas oxidation was assessed by the formation of carboxyls (Table I) as described previously (22). Western blotting confirmed that exposure to SIN-1 and MPO/H₂O₂/NO₂ but not in the other controls results in nitration of tyrosine residue(s) in all three fibrinogen chains. The nitration of fibrinogen did not result in any significant change in fibrinogen structure as determined by the CD spectrum of the control and nitrated fibrinogen (data not shown). Under our experimental conditions, exposure of fibrinogen to either SIN-1 or MPO/H₂O₂/NO₂ did not result in covariant protein cross-linking.

Scanning Electron Microscopy—Fibrin clots made at room temperature using 0.1 unit/ml thrombin were processed for scanning electron microscopy as described previously (23). All samples were prepared at least in duplicates, and for all clots at least three high resolution images from different regions were obtained.

Permeation and Viscoelastic Measurements—The Darcy constant, which represents the surface of the gel allowing flow through a network, and thus provides information on the pore structure and fiber diameter, was measured from the flow cell width, h, the pressure drop across the gel, ΔP, and the flow rate, q. The formula for the calculation of the Darcy constant, k, was k = qΔP/(h²Δh), where Δh is the height of the gel and q is the flow rate (24). Viscoelastic measurements were performed in clots prepared between 12-mm-diameter glass coverslips in a Flakz torsion pendulum described above for permeation studies (24). Each clot was measured three times, and three clots were prepared for each sample on 2 different days.

Lysis of Fibrin Clot—Clot lysis by 6 nM of recombinant tPA (American Diagnostica, Greenwich, CT) was monitored as described (25, 26). The percent lysis was calculated from the absorbance at 405 nm over time using a Hewlett-Packard spectrophotometer. The lysis of fibrin clots was confirmed by using a universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

RESULTS

Detection of Nitrated Fibrinogen in Patients with Coronary Artery Disease—Plasma of coronary artery disease patients was immunoprecipitated with a monoclonal anti-nitrotyrosine antibody. Probing the immunoprecipitated proteins with a polyclonal anti-fibrinogen antibody revealed that a fraction of fibrinogen was nitrated in coronary artery disease patient plasma (Fig. 1A). In order to confirm and quantify the presence of nitrated fibrinogen in coronary artery disease and age-matched control patient plasma, fibrinogen was purified using affinity chromatography in which the Fe portion of a polyclonal anti-fibrinogen antibody was coupled to protein A. The ability of the anti-fibrinogen column to capture nitrated fibrinogen was confirmed by using in vitro nitrated fibrinogen (not shown). The affinity column was efficient in capturing all fibrinogen for the input plasma (Fig. 1A). The eluted fibrinogen...
was then hydrolyzed in order to quantify the protein 3-nitrotyrosine levels by HPLC with on-line electrospary ionization/tandem mass spectrometry using stable isotope dilution methodology and an ion trap mass spectrometer (17, 19). The values were normalized to the levels of tyrosine to avoid changes in the measurement conditions using SIN-1 in the presence of CO2 as compared with control or fibrinogen exposed to SIN-1 in the presence of superoxide dismutase (Fig. 2C). In these experiments similar to the observations in coronary artery disease plasma, the lag phase in fibrin polymerization decreased, the maximum rate of turbidity for the first 500 s after the lag phase increased (Table 1), and the final turbidity was greater than in the control curves, suggesting alterations of clot structure. Moreover, the maximum rate of turbidity for the first 500 s after the lag phase corrected for the final turbidity increased as a function of the magnitude of fibrinogen nitration (inset, Fig. 2B). The increase in the maximum rate of turbidity of fibrinogen exposed to nitration conditions was sustained even in the presence of oxidized fibrinogen. Fibrinogen that was exposed first to SIN-1 and further oxidized by addition of 100 μM HOCl or fibrinogen that was exposed first to 100 μM HOCI and then to SIN-1 showed the same increase in maximum rate of turbidity as fibrinogen exposed only to SIN-1 (Fig. 3D). In contrast fibrinogen exposed to either MPO plus H2O2 or nitric oxide plus H2O2 or reagent HOCI failed to show increases in the maximum rate of turbidity (Fig. 2).

The significant increase in the maximum rate of turbidity was not due to acceleration in thrombin cleavage, since quantification of fibrinopeptide A and fibrinopeptide B released by thrombin proteolysis was the same between control and nitratated fibrinogen (Fig. 3).

**Factor XIII Cross-linking**—Fibrinogen cross-linking factor XIII cross-links both fibrinogen and fibrin. Fibrinogen cross-linking by factor XIII is not different between control and nitratated fibrinogen (not shown). However, factor XIII cross-linking of fibrin was accelerated in the nitrated fibrinogen than in control as evident by the disappearance of the αδ and γ chains of fibrinogen (Fig. 4).

**Effect of Nitration on Fibrin Clot Architecture, Permeation, and Viscoelastic Properties**—The architecture of the fibrin clots formed by control, nitrated, and oxidized fibrinogens was examined by scanning electron microscopy as described previously (23). Fibrin clots formed by fibrinogen exposed to nitrating agents are strikingly different from control or oxidized fibrinogens under identical experimental conditions (Fig. 5). The clots made from fibrinogens exposed to either MPO/H2O2/NO2 or SIN-1 are made up of thinner fibers, even though the higher turbidity suggested that the fibers would be thicker. However, the fibrin clot made by nitrated fibrinogens is composed of large bundles of the thin fibrin fibers, which scatter light like thick fibers, accounting for the greater turbidity. Higher magnification images also reveal the existence of twisted fibers in nitrated fibrin. A number of large pores are evident in fibrin clots made of nitrated fibrinogens in contrast to the more dense and uniform fibrin network of the oxidized fibrinogen (Fig. 5).

These changes in the architecture of the fibrin clots would be expected to have certain consequences on the physical properties of the clot. Therefore, quantitative changes in the permeation coefficient or Darcy constant were determined by flow measurements made under a constant pressure applied and

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*Fig. 1.* A. immunoprecipitated plasma proteins with affinity-purified monoclonal anti-nitrotyrosine proteins were probed with anti-fibrinogen antibodies. Lanes 1–3, three different patients with clinically documented coronary artery disease; lane 4, plasma of age-matched control patient spiked with 20 ng of nitrated fibrinogen. B, plasma from a coronary artery disease patient was passed though an anti-fibrinogen polyclonal antibody column followed by Western blotting with anti-fibrinogen monoclonal antibody. Lane 1, input plasma; lane 2, unbound fraction; lane 3, fibrinogen eluted from the column; lane 4, fraction after fibrinogen elution. C, quantification of 3-nitrotyrosine in affinity-purified fibrinogen from age- and gender-matched control (n = 26) and coronary artery disease (n = 30) patient plasma. Box-whisker plots of 3-nitrotyrosine levels in fibrinogen versus coronary artery disease status. Boxes encompass the 25th and 75th percentiles and lines within boxes represent median values. Bars represent the 2.5th and 97.5th percentiles.
accounting for geometric parameters of the clot (24). The permeation coefficient of clots made from fibrinogens exposed to either MPO/H$_2$O$_2$/NO$_2$ or H$_2$O$_2$ was significantly higher than the control (Table II). This increase in permeation is accounted for by the presence of large pores, as seen by scanning electron microscopy. A decrease in the Darcy constant was noted in the fibrin clots made of oxidized fibrinogens (Table II), since the pore size in these clots was decreased as compared with control.

Clot rigidity was then evaluated by measurement of the viscoelastic properties of the fibrin clots made from nitrated or oxidized fibrinogen. Both fibrinogens exposed to nitrating and oxidizing conditions produced clots that were considerably less stiff than control reflected by the decrease in storage modulus $G'$ value that is directly related to the stiffness of the clot (Table II). There were no apparent changes in the loss modulus $G''$ or inelastic deformation, but the ratio of inelastic to elastic deformation ($\tan\delta$) was considerably greater for clots made from both fibrinogens exposed to nitrating and oxidizing conditions (Table II).

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**TABLE I**

| Treatment of fibrinogen | $V_0$ (s$^{-1}$) $\times 10^{-4}$ | $\mu$mol nitrotyrosine/mol tyrosine | mol of carbonyl/mol of fibrinogen |
|-------------------------|---------------------------------|-----------------------------------|----------------------------------|
| MPO + H$_2$O$_2$/NO$_2$ | 23.3 ± 1.9$^a$                  | 65 ± 8                             | 0.71 ± 0.36$^a$                  |
| MPO + H$_2$O$_2$        | 0.42 ± 0.10$^a$                 | ND                                | 1.54 ± 0.45$^a$                  |
| 100 µM SIN-1            | 10.9 ± 1.0$^a$                  | 46 ± 4                            | 0.47 ± 0.07                      |
| 100 µM + SOD            | 1.19 ± 0.59                     | ND                                | 0.50 ± 0.1                       |
| Control                 | 1.03 ± 0.42                     | ND                                | 0.44 ± 0.08                      |

$p < 0.05$ after ANOVA using Tukey’s post hoc test.

The rate of clot formation ($V_0$) starting 500 s after the initiation of the reaction by the addition of thrombin was measured by turbidity changes at 350 nm ($n = 3$–5). Following the different exposures, the nitration of fibrinogen was measured by liquid chromatography/electrospray ionization/mass spectrometry analysis, whereas oxidation was assessed by the formation of carbonyls as described previously (25). ND, not detected; SOD, superoxide dismutase.

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**Fig. 2.** Thrombin-catalyzed fibrin clot formation monitored by turbidity changes at 350 nm over time after the addition of thrombin at ambient temperature. A, turbidity changes of plasma samples reconstituted with 12 mM calcium. Tracings 1–3 represent different plasma samples from coronary artery disease patients, and tracings 4 and 5 are different control plasma samples. B, turbidity changes for fibrinogen exposed to 60 nM MPO plus 100 µM H$_2$O$_2$ and 100 µM NO$_2$ (tracing 1) and fibrinogen exposed to 60 nM MPO plus 100 µM H$_2$O$_2$ (tracing 2). Inset, linear relationship between levels of in vitro nitration of fibrinogen and ratio of $V_0$/$A_{\max}$. C, turbidity changes for fibrinogen exposed to 100 µM SIN-1 (tracing 1), 100 µM SIN-1 in the presence of superoxide dismutase (tracing 2), and control fibrinogen (tracing 3). D, changes in fibrinogen turbidity after addition of thrombin to fibrinogen that was exposed first to 100 µM SIN-1 and then oxidized by the addition of more than 100 µM HOCl (tracing 1) and to fibrinogen that was first oxidized by 100 µM HOCl and then exposed SIN-1 (tracing 2). Tracing 3, control fibrinogen; tracing 4, fibrinogen exposed to 100 µM HOCl. Extensive oxidation of fibrinogen by the addition of more than 100 µM HOCl results in extensive protein cross-linking and renders the fibrinogen incapable of forming clots.

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**Table I**

Rate of clot formation of control fibrinogen and fibrinogens exposed to nitrating and oxidizing conditions

| Treatment of fibrinogen | $V_0$ (s$^{-1}$) $\times 10^{-4}$ | $\mu$mol nitrotyrosine/mol tyrosine | mol of carbonyl/mol of fibrinogen |
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$p < 0.05$ after ANOVA using Tukey’s post hoc test.
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both the control and nitrated fibrinogen. Moreover, plasmin digestions of fibrin clots made from control or fibrin made from fibrinogen exposed to nitration conditions were cleaved to the same products yielding the predicted YY, DY, DD, and E fragments (Fig. 6B). The lysis rate between the control and fibrinogen clots made from fibrinogen exposed to nitration conditions in vitro was the same after normalizing the data to the same starting turbidity and expressing it as percent lysis over time. The value for control clots was 0.10 ± 0.03 × 10−2 s−1 (n = 5) and 0.12 ± 0.02 × 10−2 s−1 (n = 5) for clots made from fibrinogen exposed to nitrating agents, suggesting that the alterations in the physical properties of the fibrin clots induced by nitration do not significantly impact the proteolytic cleavage of the clots. The in vitro data were further validated in vivo by measuring the rate of clearance of microemboli made from control and nitrated fibrinogens after injection of radiolabeled microemboli (125I-ME) with a mean diameter 2–5 μm in the tail vein of anesthetized mice (Fig. 6). As described previously, 125I-ME rapidly lodge in the pulmonary vasculature after intravenous injection in mice and rats alike and, nearly 30 min after pulmonary deposition, undergo spontaneous dissolution that is completed within 5 h (25, 26). Experiments in mice with genetically altered background revealed that both endogenous tissue type and urokinase plasminogen activator contribute to spontaneous dissolution of 125I-ME lodged in the pulmonary vessels. Injection of exogenous plasminogen activators (e.g. activase and tPA) markedly accelerates dissolution of 125I-ME deposited in rat and murine lungs and compensates for genetic ablation of the corresponding plasminogen activator (27, 28). There was no significant difference in pulmonary deposition and rate of spontaneous dissolution of 125I-ME prepared from intact versus nitrated fibrinogen in mice (Fig. 5C). However, 125I-ME prepared from nitrated fibrinogen displayed markedly higher susceptibility to “therapeutic” fibrinolysis (inset in Fig. 6C). Thus, intravenous injection of a marginally effective dose of tPA (30 μg/kg), which had no effect on the rate of dissolution of normal 125I-ME, induced a detectable acceleration of dissolution of the nitrated 125I-ME (p < 0.05). These data imply that fibrinogen nitration does not alter susceptibility of blood clots to spontaneous fibrinolysis, yet may be more amiable to fibrinolytic therapy.

Platelet Aggregation and Binding—The effects of control and nitrated fibrinogens upon the rate of human platelet aggregation and platelet binding were examined utilizing protocols established previously (29). The rate of ADP-induced platelet aggregation was the same for control and fibrinogens exposed to 100 μM SIN-1 (data not shown). Similarly, the adherence of platelets to immobilized fibrinogen on multiwell plates was the same for control and nitrated fibrinogens (data not shown).

**DISCUSSION**

A number of studies have provided evidence for a strong association between the pro-thrombotic state and risk for adverse outcomes such as myocardial infarction, sudden death, and stroke in coronary artery disease patients (1–4). Induction of a pro-thrombotic state has been associated with increased circulating levels of fibrinogen and other hemostatic proteins (1–4). Recently, it was shown that the plasma levels of nitrotyrosine, a protein marker of nitric oxide-derived oxidants, are enhanced in the plasma of coronary artery disease patients and independently predict cardiovascular risks and atherosclerotic burden (17). The present studies provide a possible biochemical link between thrombosis and inflammation by showing both increased levels of nitrated fibrinogen in coronary artery dis-
Exposure of fibrinogen to nitration conditions significantly increased, whereas oxidation significantly decreased, the permeation of clots as compared with control reflected by the changes in the Darcy constant. The Darcy constant was determined in three clots for each condition at two different pressures on 2 different days. Exposure to nitration and oxidation conditions decreased the storage modulus $G'$ forming clots that were less stiff than control clots. The viscoelastic properties of each clot were evaluated in triplicate on three separate clots for each condition on 2 different days.

|                        | Darcy constant $K_s$ cm$^2 \times 10^{-10}$ | $G'$        | $G''$      | $\tan \delta$ |
|------------------------|--------------------------------------------|-------------|------------|---------------|
| Control                | 7.5 ± 2.2                                  | 143.7 ± 38  | 10.5 ± 1.9 | 0.074 ± 0.01  |
| SIN-1                  | 584 ± 70°                                  | 42.7 ± 11°  | 9.1 ± 4.4  | 0.205 ± 0.07° |
| MPO/H$_2$O$_2$/NO$_2^-$ | 1459 ± 259°                                | 45.3 ± 9.8° | 6.2 ± 2.3  | 0.136 ± 0.03° |
| MPO/H$_2$O$_2$          | 0.9 ± 0.4°                                 | 36.5 ± 5.3° | 5.0 ± 1.4° | 0.134 ± 0.03° |

* $p < 0.05$ after analysis of variance using Tukey’s post hoc test.

FIG. 6. A, plasmin protection assay. Control and exposed fibrinogen (lanes 1 and 4) were incubated with plasmin (lanes 2 and 3 and 5 and 6) in the presence of EDTA (lanes 2 and 5) or CaCl$_2$ (lanes 3 and 6). Proteins were separated on a 7% SDS-PAGE and blotted with an antibody to fibrin degradation. Control (lane 1) and fibrin clots made from fibrinogen exposed to 60 µM MPO plus 100 µM H$_2$O$_2$ and 100 µM NO$_2^-$ (lane 2) were digested for 1 h, and the digested materials indicating the location of different fragments were separated on 4–12% gradient gels stained with colloidal blue. B, kinetics of microemboli dissolution in mice following injection of 125I-fibrin microparticles. Inset, dissolution of microparticles after administration of tPA (values indicate the number of particles in the lung at initiation and 60 min after addition of tPA (white bars) or saline (hatched bars)). Values represent mean ± S.D. of n = 3–5 independent determinations.

FIG. 2. A, plasmin protection assay. Control and exposed fibrinogen (lanes 1 and 4) were incubated with plasmin (lanes 2 and 3 and 5 and 6) in the presence of EDTA (lanes 2 and 5) or CaCl$_2$ (lanes 3 and 6). Proteins were separated on a 7% SDS-PAGE and blotted with an antibody to fibrin degradation. Control (lane 1) and fibrin clots made from fibrinogen exposed to 60 µM MPO plus 100 µM H$_2$O$_2$ and 100 µM NO$_2^-$ (lane 2) were digested for 1 h, and the digested materials indicating the location of different fragments were separated on 4–12% gradient gels stained with colloidal blue. B, kinetics of microemboli dissolution in mice following injection of 125I-fibrin microparticles. Inset, dissolution of microparticles after administration of tPA (values indicate the number of particles in the lung at initiation and 60 min after addition of tPA (white bars) or saline (hatched bars)). Values represent mean ± S.D. of n = 3–5 independent determinations.
molecules may "cap" the ends of growing protofibrils, preventing or retarding further growth. Kinetic modeling studies of changes in the rate constants resulting from capping that we have carried out are consistent with the observed effects on the polymerization curves (22). In addition, such effects could be present with only a few modified molecules as is the case with nitrated fibrinogen.

The viscoelastic properties of fibrin clots have been associated with complications of coronary heart disease (36–39). Higher fibrinogen levels have been associated with formation of a less deformable fibrin clot (clot with increased stiffness), which is more likely to occlude blood flow than a more deformable clot (36). Two studies by Fatah et al. (38, 39) described the in vitro formation of fibrin gels, made from patient plasma with documented heart disease, with abnormal gel network characterized by less porous and resilient and more space-filling structures. The biochemical reasons for the formation of these abnormal fibrin gels is different from the clots obtained from nitration selectively alters fibrinogen function, promoting clot acceleration, may account for the association between nitration of fibrinogen and the incidence of adverse effects in coronary artery disease.

Acknowledgment—We are grateful to Dr. Andrew Gow for discussions and reading the manuscript.

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