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Fibrinogen-Dependent Signaling in Microvascular Erythrocyte Function: Implications on Nitric Oxide Efflux

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Abstract Experimental evidence has shown that plasma fibrinogen plays a key role as a major cardiovascular risk factor, acting directly to trigger erythrocyte aggregation in occlusive vascular disease. However, due to the complex and hitherto unclear interaction between fibrinogen and the erythrocyte membrane, no study has yet evaluated the effects of fibrinogen, under physiological range values, on the erythrocyte nitric oxide (NO) mobilization. Taking into consideration the potential NO-derived molecules, we have raised the hypothesis that fibrinogen, under physiological conditions, may act to influence blood flow via erythrocyte NO modulation. In this in vitro study whole-blood samples were harvested from healthy subjects, erythrocyte suspensions were incubated in the absence (control aliquots) and presence of different fibrinogen concentrations and levels of NO, nitrite, nitrate and *S*-nitroglutathione (GSNO) were determined. Our results showed, when compared with control aliquots, that the presence of fibrinogen modulates the NO mobilization in erythrocytes by (1) decreasing erythrocyte NO efflux levels ($P < 0.001$); (2) increasing levels of intraerythrocytic NO oxidative metabolites, namely, nitrite ($P < 0.0001$) and nitrate ($P < 0.0001$); and (3) enhancing the formation of GSNO ($P < 0.001$). In conclusion, this study provides new insights into an unknown mechanism by which fibrinogen modulates the erythrocyte capacity to supply NO, the effects of which on inflammation profiles (generally associated with blood hyperviscosity and hyperaggregation) still need to be elucidated. Also, increased erythrocyte GSNO levels may be associated with platelet NO metabolism, its activation status and hypotension, which may be extremely relevant in the clinical setting as biomarkers.

Keywords Erythrocyte · Fibrinogen · Nitric oxide · Nitrate · Nitrite · *S*-nitrosoglutathione

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Introduction

Fibrinogen is a plasma protein which encompasses an array of functions, such as in hemostasis, cell adhesion and inflammation. It behaves, as well, as a hemorheological factor by promoting the formation of erythrocyte aggregates (Pearson and Lipowsky 2004; Grieninger et al. 1978).

The hyperaggregation state induced by fibrinogen takes place in various metabolic and cardiovascular diseases such as diabetes, arterial hypertension and atherosclerosis. Moreover, a linkage between erythrocyte adhesiveness and plasma fibrinogen levels (under inflammatory conditions) has been documented in subjects with atherosclerotic risk factors. Inhibition of erythrocyte aggregation can prevent and reduce the overall thromboembolic risk. The erythrocyte adhesiveness/aggregation test (EAAT) was used to reveal the presence of both subclinical smoldering inflammation and morbid biology in individuals with atherosclerosis risk factors (Rotstein et al. 2002). Angiographic studies have shown that high plasma fibrinogen is associated with vascular occlusion phenomena and is, thus, intrinsically involved in the physiopathology of coronary heart disease, acute myocardial infarction and stroke (ECAT Angina Pectoris Study 1993). Furthermore, it is

regarded as a major cardiovascular risk factor (Rotstein et al. 2002; Fatkin et al. 1997). Our study group has proved that erythrocyte aggregation is an independent predictor of recurrent cardiovascular events following transmural myocardial infarction (Sargento et al. 2005).

It has been suggested that the fibrinogen 455G/A, but not the normal genotype (Ben Assayag et al. 2006), is associated with erythrocyte hyperaggregation (Weng et al. 1999). Recently, a large-scale proteomic analysis disclosed an association between fibrinogen and hypercoagulability states in pancreatic cancer (Bloomston et al. 2006).

Prospective studies in healthy men (Meade et al. 1993; Wilhelmsen et al. 1984) and women (Kannel et al. 1987) have shown that a single fibrinogen measurement predicts fatal and nonfatal cardiovascular events as much as 16 years later. The fibrinogen level predicts restenosis after angioplasty (Montalescot et al. 1995), and it may promote, along with other haemostatic factors, atherosclerotic changes and thrombosis by mechanisms shown in vitro on platelet aggregability, blood viscosity and foam cell formation. An alternative view is that the eventual fibrinogen–cardiovascular disease association may be a consequence, rather than a cause, of the disease process, perhaps due to an inflammatory response to progressive endothelial damage. This view identifies fibrinogen as a marker of long-term physiopathological changes. Both perspectives, which are certainly not mutually exclusive, support the use of fibrinogen as a cardiovascular risk factor in epidemiological studies (Brunner et al. 1996).

Endothelium-derived nitric oxide (NO) consists of a further molecule important in the control of blood flow and pressure (Sonveaux et al. 2007). Vascular NO has been identified as having a key role in blood pressure regulation. It lowers blood pressure by stimulating the release of calcium from vascular smooth muscle cells, thereby causing the blood vessels to dilate (Gladwin et al. 2004). There is evidence that NO deficiency can cause hypertension and may also be involved in the pathogenesis of atherosclerosis. Uncontrolled NO production, however, can lead to massive peripheral vasodilation and shock (Drexler 1997).

Following its passage into red blood cells by simple random diffusion, NO may either be stored or return to the bloodstream as an active *S*-nitrosothiol molecule. It interacts with several components of the erythrocyte membrane, as well as with hemoglobin (Pawloski and Stamler 2002; Pawloski et al. 2001; Gross and Lane 1999). The major stable metabolites resulting from NO oxidation, represented by NO_x, include nitrites (NO_2^-) and nitrates (NO_3^-). NO furthermore reacts with superoxide anion to yield peroxynitrite, which may either yield nitrates or damage proteins, lipids and carbohydrates via oxidation and nitration reactions. Glutathione, an antioxidant molecule with a thiol group, binds to NO, yielding *S*-nitrosoglutathione (GSNO), a secondarily important storage molecule (Tarpey and Fridovich 2001). Considering the fact that all degrees of inflammation are associated with reactive oxygen and nitrogen species, hyperfibrinogenemia and hyperaggregation states, we might raise a hypothesis concerning possible concentration changes in the erythrocyte NO scavenger and derivative molecules.

No study has yet been conducted to evaluate the effect of plasma fibrinogen on erythrocyte NO metabolism and mobilization. In view of this, we assessed the in vitro influence of fibrinogen on erythrocyte NO efflux, nitrites, nitrate and GSNO concentrations.

Methods

Chemicals

The chosen concentrations for fibrinogen were based on its physiological levels and previous studies (Sargento et al. 2005). Human fibrinogen was purchased from Sigma (Poole, UK).

Preparation of Erythrocyte Suspensions

Human venous blood samples were collected from the forearm vein of 15 healthy Caucasian men after informed consent. The blood container tubes were prepared with 10 IU ml^{-1} of sodium heparin (anticoagulant). The blood was centrifuged at $1{,}040\times g$ for 10 min in a Sorvall (Wilmington, DE) TC6 centrifuge. Afterward, the plasma and buffy coat (leukocytes and platelets) were discarded. Erythrocyte suspensions were performed, after previous passage through an Imugard IG 500 column (Terumo, Tokyo, Japan), with the addition of sodium chloride (0.9% at pH 7.4, AnalaR; BDH Laboratory, Poole, UK), to reconstitute the initial hematocrit (Ht $\sim$45%).

Experimental Design

Erythrocyte suspension aliquots were then incubated for 30 min at room temperature, in the absence and presence of fibrinogen increasing concentrations (150, 250, 300 and 400 mg/dl). All erythrocyte suspensions were incubated in slight agitation.

Measurement of NO by an Amperometric Method

Following incubation, erythrocyte suspensions were centrifuged and sodium chloride 0.9% at pH 7.0 was added in order to compose a hematocrit of 0.05%. The suspension was mixed by gentle inversion of tubes.

For amperometric NO quantification, we used the amino-IV sensor (Innovative Instruments, Tampa, FL), a

method previously described by us (Carvalho et al. 2004a). NO diffuses through the gas-permeable membrane triple COAT of the sensor probe and is then oxidized at the working platinum electrode, resulting in an electric current. The redox current is proportional to the NO concentration outside the membrane and is continuously monitored with the inNO model T electrochemical detection system (version 1.9, Innovative Instruments) and connected to a computer. Calibration of the NO sensor was performed daily. For each experiment, the NO sensor was immersed vertically in the erythrocyte suspension vials and allowed to stabilize for 30 min to achieve NO basal levels. Acetylcholine (Ach, 30 µl; Sigma, St. Louis, MO) was added to the erythrocyte suspension aliquots in order to achieve a final concentration of 10 µM, and the NO released from erythrocytes was registered. Data were recorded from constantly stirred suspensions at room temperature.

Measurement of Nitrite/Nitrate Concentration Using the Spectrophotometric Griess Method

After the incubation period, the erythrocyte suspensions, performed as described above, were centrifuged at 9,600×*g* for 1 min using the Biofuge 15 (Heraeus, Wehrheim, Germany) centrifuge. The supernatants were then separated from the pellet (packaged erythrocytes).

Nitrite and nitrate levels in the intraerythrocyte compartment were determined as previously described by us (Carvalho et al. 2004b), after submitting the pellet of each suspension to hemolysis and hemoglobin precipitation (erythrocyte cytoplasm values registered). Hemolysis was induced with distilled water and hemoglobin precipitation with ethanol and chloroform.

Nitrite concentrations were measured with the spectrophotometric Griess reaction, at 548 nm. For nitrate measurement, this compound was first reduced to nitrites in the presence of nitrate reductase (Guevara et al. 1998).

Measurement of GSNO

Colorimetric solutions containing a mixture of sulfanilic acid (B component of Griess reagent) and NEDD (A component of the Griess reagent), consisting of 57.7 mM sulfanilic acid and 1 mg/ml NEDD, were dissolved in phosphate-buffered saline (PBS), pH 7.4. To constitute the 10 mM $HgCl_2$ (Aldrich, Milwaukee, WI) mercury ion, stock solutions were prepared in 0.136 g/50 ml dimethyl sulfoxide (DMSO, Aldrich). GSNO was diluted to the desired concentration in the colorimetric analysis solutions. Various concentrations of mercury were then added to a final concentration of 100 µM. Following gentle shaking, the solution was left to stand for 20 min. A control spectrum was measured by spectrophotometry at 496 nm against a solution without mercury ion. Erythrocyte suspensions (300 µl) were added to the reaction mixture, and GSNO concentrations were obtained as described by Cook et al. (1996).

Statistical Analysis

Data are expressed as means ± SD. Student's paired *t*-tests were used to compare values between different aliquots of erythrocyte suspensions. Statistical analysis was conducted using the Statistical Package for the Social Sciences software, version 16.0 (SPSS, Inc., Chicago, IL). One-way analysis of variance tests and paired *t*-tests were applied to assess statistical significance among samples. Bonferroni post-hoc tests were conducted when appropriate. Statistical significance was set at $P < 0.05$.

Results

Effects of Fibrinogen Concentrations on NO Levels

Statistically significantly lower values were found for the erythrocyte NO efflux measured in aliquots incubated with fibrinogen concentrations of 150, 250, 300 and 400 mg/dl, when compared with erythrocyte suspension aliquots without fibrinogen.

The NO values are shown in Fig. 1. In control aliquots the NO was 2.28 ± 0.27 nM, which was higher than the values obtained in aliquots incubated in the presence of (1) fibrinogen 150 mg/dl, where the NO concentration was 1.56 ± 0.17 nM ($P < 0.0001$ vs. control); (2) fibrinogen 250 mg/dl, with an average concentration of 2.01 ± 0.22 nM ($P < 0.05$ vs. control); (3) fibrinogen 300 mg/dl, with an average concentration of 1.90 ± 0.31 nM ($P < 0.05$ vs. control); and (4) fibrinogen 400 mg/dl, with NO levels of 1.87 ± 0.09 nM ($P < 0.005$ vs. control).

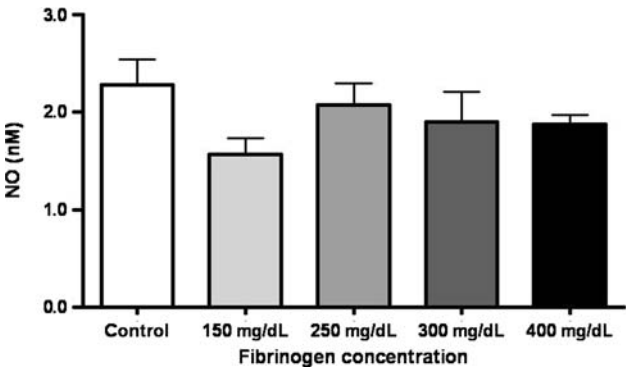

Fig. 1 Effects of fibrinogen concentrations on NO levels in erythrocyte suspensions. Values are mean ± SD ($n = 15$)

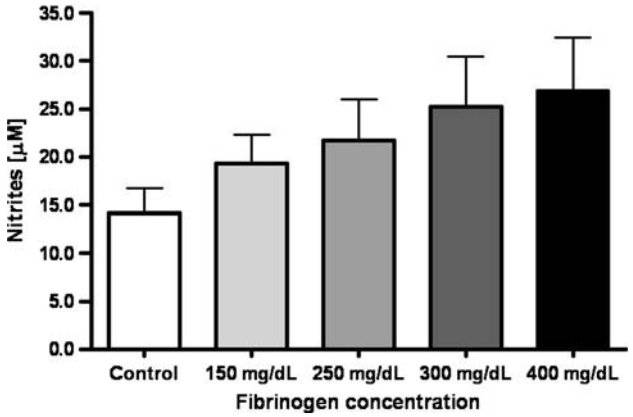

Fig. 2 Changes in nitrite levels in erythrocyte suspensions incubated with increasing fibrinogen concentrations. Values are mean ± SD ($n = 15$)

Effects of Fibrinogen Concentrations on Erythrocyte Nitrite and Nitrate Levels

There was a statistically significant increase in nitrite/nitrate levels of erythrocyte suspensions in the presence of all fibrinogen concentrations used, when compared with the erythrocyte suspension aliquots without fibrinogen (Figs. 2, 3).

To specify, (1) the presence of fibrinogen 150 mg/dl disclosed an average of 19.6 ± 2.95 µM vs. control 13.90 ± 2.57 µM ($P < 0.005$) for nitrite and 20.40 ± 2.23 µM vs. control 14.60 ± 2.34 µM ($P = 0.001$) for nitrate concentrations; (2) the presence of fibrinogen 250 mg/dl showed an average concentration of 21.85 ± 4.51 µM ($P < 0.0001$ vs. control) for nitrite and 22.7 ± 4.67 µM ($P < 0.0001$ vs. control) for nitrate concentrations; (3) with fibrinogen 300 mg/dl we observed an average concentration of 24.90 ± 5.42 µM ($P < 0.0001$ vs. control) for nitrite and 25.05 ± 5.47 µM ($P < 0.0001$ vs. control) for nitrate concentrations; and (4) with fibrinogen 400 mg/dl we observed

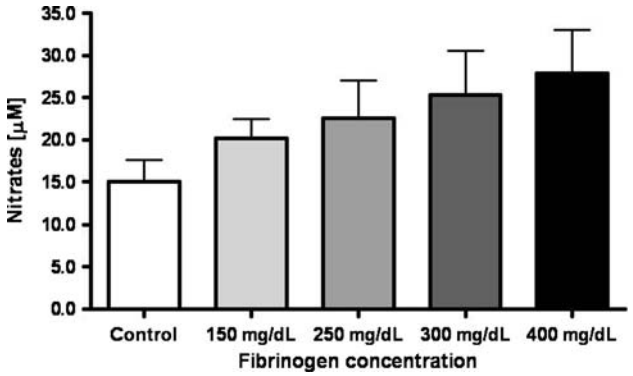

Fig. 3 Changes in nitrate levels in erythrocyte suspensions incubated with increasing fibrinogen concentrations. Values are mean ± SD ($n = 15$)

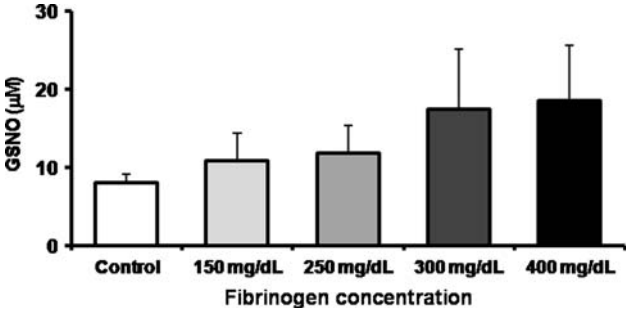

Fig. 4 Effects of increasing fibrinogen concentrations on GNSO levels in erythrocyte suspensions. Values are mean ± SD ($n = 15$)

an average concentration of 27.80 ± 4.87 µM ($P < 0.0001$ vs. control) for nitrite and 28.70 ± 4.55 µM ($P < 0.0001$ vs. control) for nitrate concentration.

Effects of Fibrinogen Concentrations on Erythrocyte GSNO

Statistically significantly higher values were found for erythrocyte GSNO after incubation in the presence of different fibrinogen concentrations compared with aliquots without fibrinogen, as shown in Fig. 4. When erythrocyte suspension aliquots were incubated in the presence of fibrinogen concentrations of 150, 250, 300 and 400 mg/dl, the erythrocyte GSNO concentrations were, respectively, 10.86 ± 3.46 µM ($P = 0.03$), 11.85 ± 3.5 µM ($P = 0.003$), 17.45 ± 7.59 µM ($P = 0.001$) and 18.46 ± 7.08 µM ($P < 0.0001$), which are higher than the value obtained in the absence of fibrinogen (control aliquot), 7.95 ± 1.13 µM.

Discussion

To the best of our knowledge, this study is the first to demonstrate that fibrinogen can modulate erythrocyte NO mobilization through an as yet unknown signaling pathway. The main findings concerning erythrocyte suspension aliquots incubated with different fibrinogen concentrations were the decrease of NO efflux, increase of its oxidative molecules such as nitrite and nitrate and higher levels of NO scavenger molecules such as GSNO. These results may be explained by the intraerythrocyte NO reactions from its well-known reservoir molecules and via metabolic reactions, as a way to redistribute NO-derived molecules (Huang et al. 2001). No linear dose-dependent response to fibrinogen was verified regarding NO efflux, despite significantly lower values obtained in the control aliquots. Besides the higher values of nitrites and nitrates obtained in relation to the control aliquots, a nonlinear dose-dependent effect of fibrinogen on nitrite/nitrate and GSNO levels was observed, without any changes in osmolality upon

fibrinogen loading (data not shown). These results may indicate either an inside–out or an outside–in signal-transduction mechanism encompassing conformational changes in the protein and participants. Furthermore, it has been formerly shown by others that erythrocyte NO flux is mediated by band 3 protein, with no efflux occurring when methemoglobin and deoxygenated hemoglobin bind to the cytoskeleton (Huang et al. 2001). In view of this, a signal-transduction mechanism associating plasma fibrinogen with band 3 protein may be hypothesized in order to explain the lower erythrocyte ability to release NO, in conjunction with the increased intraerythrocyte amount of nitrites, nitrates and GSNO. We have also documented that ACh increases the erythrocyte NO efflux and signal-transduction pathways influenced by band 3 and dithiothreitol (Carvalho et al. 2006, 2008; Almeida et al. 2008).

We have demonstrated that when stimulating the erythrocyte redox thiol status by dithiothreitol loading, there is a decreased NO efflux concomitant with increased levels of nitrite, nitrate and GSNO (Lopes de Almeida et al. 2009). It is well known that dithiothreitol induces band 3 dephosphorylation (Zipser et al. 1997), and a dephosphorylated state may hypothetically account for the fibrinogen effects on red cells.

This erythrocyte mechanism mediated by fibrinogen may be important, e.g., since thiol molecules, especially glutathione, act to supply the organism with a variety of functions in human metabolism, detoxification of xenobiotics, cell homeostasis, radioprotection and antioxidant protection required in inflammatory states. *S*-Nitrosothiols are biological metabolites derivatives from NO (Doctor et al. 2005). It has often been suggested that they represent more stable metabolites of NO, which can be either stored or transported, although this evidence is sparse. The erythrocytic retention of *S*-nitrosothiols throughout the circulatory transit was reported in the past, by several authors, as a significant way to prevent pathological vasodilation (McMahon et al. 2000).

NO itself is able to modulate its own consumption by red cells under hypoxic conditions (Doctor et al. 2005). In accordance, the biochemical standpoint behind the inhalation of therapeutic NO is supported by the interaction of NO with different oxygen-hemoglobin states (either oxygenated or deoxygenated), with *S*-nitrosothiol formation being limited to a lesser extent (Cannon et al. 2001). Thus, in accordance with other authors, the issue of the mechanism by which erythrocyte GSNO elicits vasodilation remains unclear (Hogg 2002; Spencer et al. 2000).

In our study, the finding that fibrinogen acts to increase GSNO levels implies that it may indirectly modulate blood flow and pressure in vivo. Glutathione is a well-known abundant molecule inside erythrocytes, and it has a thiol group which can react with NO or other molecules to yield nitrosothiols, namely GSNO (Galli et al. 2002). Furthermore, GSNO is an effective platelet activation inhibitor, considered by many to be one of the best NO donors in thrombotic illness (Radomski et al. 1992; Ramsay et al. 1995). A further animal study has demonstrated evidence that NO can tonically act to reduce plasma fibrinogen levels in rats (Kawabata 1996). This study was conducted under physiological conditions and hypothesized that NO reacts with superoxide anion and the yielding product, peroxynitrite anion, may act to decrease plasma fibrinogen levels. Taking into account that higher plasma fibrinogen levels are regarded as a marker of inflammatory states, concomitantly associated with oxidative stress and induced erythrocyte hyperaggregation, it was useful to assess whether there is an effect on erythrocyte NO efflux.

The precise mechanisms by which plasma fibrinogen interacts with the erythrocyte membrane to mobilize NO from store molecules into its oxidative metabolites (and vice versa) are unclear at present. Further studies are compulsory to deepen this topic. Different approaches have been attempted to identify the erythrocyte membrane domain responsible for fibrinogen attachment. We have verified that the erythrocyte membrane binding sites for fibrinogen protein molecules are strongly dependent on the conformational state of the protein itself (Gonçalves et al. 2006). Additional in vitro experiments were conducted with the fibrinogen concentration kept constant while adding vasoactive molecules that modify hemorheological erythrocyte properties (Hilário et al. 2003; Almeida et al. 2008; Santos et al. 2003).

In conclusion, for the first time, this study proposes the possibility that endogenous NO is modulated by plasma fibrinogen in a nonlinear, dose-dependent manner in human erythrocytes. Also, we may hypothesize that fibrinogen-triggered erythrocyte GSNO and decreased NO efflux may, if verified in vivo, be associated with coagulopathy and hypotension under acute phase states. These effects on NO-derived molecules allowing intraerythrocyte NO scavenging may be protective under inflammatory conditions.

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