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Mechanisms of fibrinogen-induced microvascular dysfunction during cardiovascular disease

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Abstract

Fibrinogen (Fg) is a high molecular weight plasma adhesion protein and a biomarker of inflammation. Many cardiovascular and cerebrovascular disorders are accompanied by increased blood content of Fg. Increased levels of Fg result in changes in blood rheological properties such as increases in plasma viscosity, erythrocyte aggregation, platelet thrombogenesis, alterations in vascular reactivity and compromises in endothelial layer integrity. These alterations exacerbate the complications in peripheral blood circulation during cardiovascular diseases such as hypertension, diabetes and stroke. In addition to affecting blood viscosity by altering plasma viscosity and erythrocyte aggregation, growing experimental evidence suggests that Fg alters vascular reactivity and impairs endothelial cell layer integrity by binding to its endothelial cell membrane receptors and activating signalling mechanisms. The purpose of this review is to discuss experimental data, which demonstrate the effects of Fg causing vascular dysfunction and to offer possible mechanisms for these effects, which could exacerbate microcirculatory complications during cardiovascular diseases accompanied by increased Fg content.

Keywords

arteriolar constriction; endothelial cell layer permeability; endothelin-1; erythrocyte aggregation; tight junction proteins; Weibel-Palade body exocytosis

Fibrinogen (Fg) is a biomarker of inflammation (Ross 1999), which, when elevated, indicates the presence of inflammation and identifies individuals with a high risk for cardiovascular disorders. Fg is synthesized and assembled in hepatocytes and fibroblasts, and when secreted into the circulation, its plasma half-life ranges from 3 to 4 days (Collen *et al*. 1972, Martinez *et al*. 1974). Increased plasma Fg concentration typically accompanies hypertension development (Letcher *et al*. 1981, Lominadze *et al*. 1998) and stroke (D'Erasmo *et al*. 1993). Other inflammatory biomarkers such as interleukin (IL)-6 (Dalekos *et al*. 1996, Nakamura *et al*. 1996, Chae *et al*. 2001) and IL-1 (Tikkanen *et al*. 1995, Kannan *et al*. 1996, Nakamura *et al*. 1996, Yudkin *et al*. 1999), which are involved in the synthesis of Fg (Humphries 1995, Vasse *et al*. 1996), are also associated with elevation of blood pressure. These data suggest

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Conflict of interest
There is no conflict of interest in this study.

that Fg overproduction is involved in progression of hypertension and may even be involved in its development.

Many demographic and environmental factors (Krobot *et al*. 1992) as well as genetic factors (De Maat 2001) determine Fg levels. While the effect of most environmental factors is largely through the acute phase reaction, estimates based on twin studies suggest that 30–50% of the plasma Fg level is genetically determined (Hamsten *et al*. 1987, Humphries *et al*. 1987, Pankow *et al*. 1998). Two independent meta-analysis studies estimated that individuals in the upper tertile of Fg concentration had a 2.3-fold greater risk of subsequent cardiovascular disease than individuals in the lower tertile (Ernst & Resch 1993, Danesh *et al*. 2005). This creates a greater interest in Fg and an increased appreciation of its importance, role and implications in cardiovascular diseases.

Recent studies indicate that increased Fg content affects microcirculation by increasing plasma viscosity, RBC aggregation and platelet thrombogenesis, altering vascular reactivity and compromising endothelial cell layer integrity. These changes lead to vascular dysfunction and exacerbate microcirculatory complications during cardiovascular diseases. The purpose of this review is to discuss the detrimental effects of elevated levels of Fg that cause vascular dysfunction (Fig. 1) and the possible mechanisms of these effects. Based on the results of our studies and studies of others, we present a new hypothesis to define a possible mechanism for Fg-induced vasoconstriction (Fig. 2). Some aspects of this mechanism need to be confirmed by further experimental data. Therefore, we hope that the discussion presented below may trigger ideas for additional experiments to clarify the mechanisms of microvascular dysfunction caused by an increased blood content of Fg.

Effects of Fg in the microcirculation

Fg and blood viscosity

Increased total peripheral vascular resistance (TPVR) is typically associated with the development of cardiovascular and cerebrovascular diseases, many of which are accompanied by increased arterial pressure. Arterial pressure is dependent on cardiac output and TPVR, with the latter being determined by the calibre of resistance vessels and the intrinsic viscosity of blood. As cardiac output is generally normal in established hypertension (Lund-Johansen 1983), it is likely that elevated TPVR is commonly the main cause of increased arterial blood pressure. Major determinants of TPVR include vessel radius (Folkow 1978, Wiegman *et al*. 1979) and number of vessels (Bohlen 1989), which are decreased during hypertension.

Blood viscosity is one of the strongest predictors of adverse cardiovascular events such as hypertension, stroke and diabetes (Lowe *et al*. 1997). Increased blood viscosity is a result of changes in a number of variables, including increased haematocrit, erythrocyte aggregation and plasma viscosity (Dintenfass 1978, Letcher *et al*. 1981, Chien 1986, Chabanel *et al*. 1987, Chabanel & Chien 1990, London 1997, Lowe *et al*. 1997). Plasma viscosity is elevated in hypertensive patients, and is positively correlated with blood pressure (Koenig *et al*. 1989). A major determinant of plasma viscosity is the Fg content. Plasma Fg levels are higher (3.8 ± 0.1 mg mL^{-1}) in patients with essential hypertension than in normotensive controls (2.8 ± 0.1 mg mL^{-1}) (Letcher *et al*. 1981). Even when hypertension is mild, Fg levels are higher than in normotensive controls (Landin *et al*. 1990).

Increased plasma Fg concentration increases blood viscosity, and therefore increases blood flow shear stress (Lowe *et al*. 1997) that activates endothelial cells (Shyy & Chien 2002, Davies *et al*. 2003) and platelets (Ruggeri 1993). Activation of endothelial cells results in expression and/or activation of various adhesion molecules (Plow *et al*. 2000) and integrins (Languino *et*

al. 1993). Some of these molecules, such as intercellular adhesion molecule-1 (ICAM-1) (Suehiro *et al*. 1997) and $\alpha_5\beta_1$ integrin (Kern *et al*. 1986), are receptors for Fg.

Red blood cell (RBC) aggregation is a major determinant that affects blood flow in resistance vessels at low shear rates by increasing blood viscosity and inducing microcirculatory sludging and stagnation in small vessels (Chien *et al*. 1967). Although several convincing studies illustrate that an increase in RBC aggregation causes a decrease in local blood flow in microvessels (Zilliacus 1951, Bloch 1956, Knisely 1965), this concept was ignored for some time. More recently, several laboratories found that an increase in erythrocyte aggregation causes an elevation in total vascular resistance to blood flow (Gustafsson *et al*. 1981a,b, Maspers *et al*. 1990, Vicaut *et al*. 1994). Cabel *et al*. (1997) showed that RBC aggregation-induced blood flow viscosity changes venous resistance to flow. In addition, blood flow resistance was increased in arterioles and capillaries of the microcirculation by RBC hyperaggregability (Vicaut *et al*. 1994, Vicaut 1995) and a role of Fg was suggested (Vicaut 1995). In fact, many cardiovascular diseases and cerebrovascular disorders are characterized by both increased RBC aggregation (Zannad & Stoltz 1992, Mchedlishvili *et al*. 1993, Lominadze *et al*. 1998, Hacioglu *et al*. 2002) and plasma viscosity (Lip 1995, Qizilbash 1995, Lowe *et al*. 1997).

Two major theoretical models have been proposed for RBC aggregation by non-specific mechanisms (Rampling *et al*. 2004). The first, based on a macromolecular bridging model, is well accepted for describing the erythrocyte rouleaux formation and is based on the surface adsorption of macromolecules to form a bridging configuration between adjacent erythrocytes (Chien *et al*. 1967, Chien & Jan 1973). van der Waal's forces, hydrogen bonds and electrostatic attractions are believed to favour the adsorption of macromolecules (Chien *et al*. 1967). The other theory suggests that aggregation is induced by macromolecular depletion from the membrane surface (Evans & Needham 1988). According to the former non-specific adhesion theory, polymers and plasma proteins with a large molecular mass packed between adjacent erythrocytes, increasing the intercellular distance and inducing erythrocyte aggregation by decreasing the electrostatic repulsive forces between erythrocytes (Chien & Jan 1973). Therefore, the increased aggregation can also be explained by an increase in the adsorption area on the cell surface due to the larger size of the macromolecules (Chien & Jan 1973). In the latter nonspecific adhesion theory, aggregation is thought to be independent of both the molecular mass and surface adsorption. However, it is well documented that RBC aggregation is promoted mainly by proteins with higher molecular weight (Zannad & Stoltz 1992, Lip & Beevers 2007). In fact, Weng *et al*. (1996) showed that plasma proteins with a large molecular mass such as haptoglobin (86–400 kDa), C-reactive protein (105 kDa), ceruloplasmin (132 kDa) and Fg (340 kDa) exhibited strong effects on erythrocyte aggregation. However, proteins with a lower molecular weight such as α_1-acid glycoprotein (40 kDa) and α_1-antitrypsin (54 kDa) played no significant role in RBC aggregation (Putnam 1984). These discrepancies between the theory and facts point to the possible co-existence of a specific binding mechanism. Indeed, contrary to the accepted hypothesis that RBCs do not have receptors for adhesion proteins, specific receptors for ceruloplasmin were identified on the erythrocyte membrane (Barnes & Frieden 1984, Saenko & Yaropolov 1990). Presently, to our knowledge, no studies have been performed to determine whether plasma adhesion proteins involved in RBC aggregation have specific binding sites on the erythrocyte membrane.

Fibrinogen has a greater effect than other plasma proteins on RBC aggregation (Letcher *et al*. 1983, Chen & Schachter 1993, Game *et al*. 1996, Weng *et al*. 1996, Lominadze *et al*. 1998). We found that Fg can specifically bind erythrocyte membranes (Lominadze & Dean 2002). In hypertensive animals, the ability of erythrocytes to aggregate (RBC aggregability) was associated with hypertension (Lominadze *et al*. 1998, 2002). In addition, the composition of the RBC membrane changes with development of hypertension (Lominadze *et al*. 1998,

2002). These data suggest that with the development of hypertension, specific Fg receptors on the RBC membrane may be altered. For example, platelets specifically bind through their $\alpha_{IIb}\beta_3$ integrin (GPIIb/IIIa) to RBC membrane ICAM-4 (Hermand *et al*. 2003), which may compound the deleterious effects of RBC aggregation on blood rheological properties by promoting thrombosis.

Fg and vasoactivity

Despite the knowledge that Fg is a high risk factor for many cardiovascular and cerebrovascular disorders (Lowe *et al.* 1997), its effect on vascular reactivity is poorly understood. The ability to stimulate frog heart by the extracted clot liquors obtained after conversion of Fg to fibrin by thrombin was first mentioned in 1951 (Laki 1951). It was then found that fibrinopeptide-B, which is obtained as a result of Fg conversion to fibrin by thrombin, can increase bradykinin-induced contraction of isolated rat uterus in the oestrus cycle (Gladner *et al*. 1963). Further studies have shown short-lasting effects of fibrinopeptides A and B on arterial blood pressure in rats (Barczak-Osinska *et al*. 1983). Vasoactive effects of isolated peptides derived from plasmin digestion of fibrin and Fg were studied in various vascular beds including lung (Kern *et al*. 1986), heart (Mehta *et al*. 1985, Nichols *et al*. 1985), femoral artery (Saldeen *et al*. 1991) and mesenteric artery (Anderson *et al*. 1983).

The vasoactive effect of Fg as an intact protein was first shown by Hicks *et al*. (1996). They found that low concentrations of Fg (up to 2 μ_M) induced endothelium-dependent dilation of isolated saphenous vein rings. A maximum relaxation to Fg of about 60% was achieved at a concentration of 1.8 μ_M (Hicks *et al*. 1996). Diameters of the isolated rings were restored to a normal level in the presence of higher concentrations of Fg (5–6 μ_M). In this study, while the role of endothelial $\alpha_V\beta_3$ integrin was ruled out, Fg binding to endothelial ICAM-1 was suggested as a possible mechanism for initiation of signalling pathways leading to the synthesis of vasoactive mediators other than nitric oxide (NO) and prostacyclin (Hicks *et al*. 1996). Recent work by others showed that increased content of Fg causes dilation of small (0.8–1.4 mm in diameter) porcine coronary artery and human internal thoracic artery segments (Bas *et al*. 2008). In this study, 2 μ_M of Fg induced vasodilation of about only 20%, while approx. 60% relaxation was achieved at an Fg concentration of 12 μ_M (Bas *et al*. 2008). Fg-induced vasorelaxations were abolished by abciximab (platelet aggregation inhibitor that inhibits platelet GPIIb/IIIa) and diminished by endothelial denudation or NO synthase inhibitors (Bas *et al*. 2008). As abciximab binds $\alpha_V\beta_3$ integrin with the same affinity as GPIIb/IIIa, the authors suggested that Fg binding to vascular $\alpha_V\beta_3$ integrin leads to the synthesis of vasoactive mediators (Bas *et al*. 2008). Discrepancies between these studies may result from differences among the types of vessels, species and the avidities (avidity is the combined synergistic strength of bond affinities, thus it relates to the number of receptors) of Fg receptors on the endothelium.

Treatment of rat pulmonary artery rings by early digestion products of Fg did not cause vasoconstriction (Boutcher *et al*. 1996). However, in this work, responses of the vascular rings to Fg degradation products were tested in the presence of 4×10^{-8} M phenylephrine (PE) which induced 50% of the maximal contraction. Therefore, PE could have masked the effects of Fg digestion products on vasoconstriction. Arterial constriction induced by Fg binding to endothelial ICAM-1 was first demonstrated by Lominadze *et al*. (2005). It was also found that this Fg-induced vasoconstriction can be reversed by endothelin type A (ET-A) receptor inhibition (Lominadze *et al*. 2005). Further studies by the same group showed that elevated content of Fg increases production of endothelin-1 (ET-1) by means of enhanced exocytosis of Weibel-Palade bodies (WPbs) from endothelial cells (Sen *et al*. 2009). Activation of extracellular signal-regulated kinase-1/2 (ERK-1/2) by Fg binding to endothelial ICAM-1 was

suggested to enhance production of ET-1 (Sen *et al*. 2009). Figure 2 presents a schematic representation of the hypothesis for the possible mechanism of Fg-induced vasoconstriction.

Other Fg receptors on endothelial cell membrane are $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins (Luscinskas & Lawler 1994). Mogford *et al*. (1996, 1997) showed that endothelial $\alpha_v\beta_3$ and $\alpha_5\beta_1$ receptors induce opposite effects on vascular tone in response to Arg-Gly-Asp-Asn (RGDN)-containing peptide. The RGDN peptide induced dilation through a vascular smooth muscle cell (VSMC) $\alpha_v\beta_3$ integrin-mediated mechanism (Mogford *et al*. 1996), whereas $\alpha_5\beta_1$ integrin mediated vascular constriction (Mogford *et al*. 1997). Arterioles denuded of a functional endothelium were unable to maintain the constriction induced by RGDN-containing peptide, indicating that the response resulted from the interaction of the RGD sequence with endothelial $\alpha_5\beta_1$ integrin (Mogford *et al*. 1997). However, luminal application of fibronectin or RGDN peptide did not alter arteriolar diameter (Mogford *et al*. 1997). Therefore, the authors concluded that the constrictor response to the RGDN peptide was mediated by abluminal endothelial $\alpha_5\beta_1$ integrin (Mogford *et al.* 1997). A role of ET-1 was suggested in the mechanism for RGD/$\alpha_5\beta_1$ integrin interaction-induced vasoconstriction (Mogford *et al.* 1997). Reduction of VSMC intercellular Ca^{2+} (D'Angelo *et al*. 1997) and K^+ channel activation (Platts *et al*. 1998) were implicated in RGD/$\alpha_v\beta_3$ integrin interaction-induced vasodilation (Mogford *et al*. 1996). The later studies by the same group showed that both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins are required to produce myogenic vasoconstriction in skeletal muscle arterioles (Martinez-Lemus *et al*. 2005).

Fibrinogen contains two RGD sequences on its Aa chain (Suehiro *et al*. 1997). The Aα chain residues 572–574 are the binding sites for both $\alpha_v\beta_3$ (Smith *et al*. 1990) and $\alpha_5\beta_1$ (Suehiro *et al*. 2000) integrins, suggesting that these integrins compete for binding to Fg. It was reported that plasma fibronectin does not bind luminal $\alpha_5\beta_1$ integrin (Zanetti *et al*. 1994, Kano *et al*. 1996). However, binding of endothelial cells to immobilized Fg occurs through $\alpha_5\beta_1$ integrin (Suehiro *et al*. 1997). In the absence of $\alpha_5\beta_1$, $\alpha_v\beta_3$ integrin exists in an activated state with high affinity for Fg (Ly *et al.* 2003). *De novo* expression of $\alpha_5\beta_1$ integrin suppresses $\alpha_v\beta_3$ integrin-mediated adhesive functions (Ly *et al.* 2003). These data suggest that there may be variations in the expression of $\alpha_5\beta_1$ during pathologies, which lead to the modulation of $\alpha_v\beta_3$ integrin affinity for its ligands (Ly *et al.* 2003). In addition, while the 120 kDa fragment of fibronectin supports $\alpha_5\beta_1$ binding, smaller fragments (<5 kDa) of the protein have greater affinity for $\alpha_v\beta_3$ integrin (Pytela *et al*. 1985). These results indicate that the functional role of $\alpha_v\beta_3$ integrin is greater if smaller protein fragments are present in the blood.

ICAM-1 was shown to have a role in Fg binding to endothelial cells *in vitro* (Porteri *et al*. 1998). As binding of Fg to ICAM-1 is independent of its RGD sequence (Suehiro *et al*. 1997, Pluskota & D'Souza 2000), these data indicate that ICAM-1 may also be involved in Fg binding to the vascular endothelium, and serve as a possible mechanism for Fg-induced vasoconstriction. This hypothesis is corroborated by studies showing that the binding affinity of Fg to ICAM-1 is higher than the binding to β_1 integrin (D'Souza *et al*. 1996, Lominadze *et al*. 2005). In addition, $\alpha_5\beta_1$ and other integrins have higher affinity for Fg only when they are in the activated state (Suehiro *et al*. 1997). These results suggest that Fg may bind ICAM-1 and $\alpha_5\beta_1$ or $\alpha_v\beta_3$ integrins simultaneously, but with different binding affinity.

From these studies it appears that $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins compete for a binding site on Fg, while ICAM-1, when activated, has no competition from integrins. The discrepancies in the results of various studies demonstrating opposite effects of Fg-induced vasoactivity may be due to differences in experimental set-ups leading to differences in the expression of Fg receptors and/or differences in the vessels used in the studies resulting in different avidities of the Fg receptors. Further studies are required to resolve the discrepancies in Fg-induced vasodilatory and vasoconstrictory effects and to dissect the mechanisms of action of endothelial ICAM-1, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin during inflammation-induced endothelial activation.

ICAM-1 and $\alpha_5\beta_1$ integrin expression and/or activation—During hypertension, which is an inflammatory cardiovascular disease (Savoia & Schiffrin 2006, Wang *et al*. 2007), mRNA expression of ICAM-1 is upregulated (Buemi *et al*. 1997, DeSouza *et al*. 1997, Porteri *et al*. 1998, Becker *et al*. 2000). Although expression of $\alpha_5\beta_1$ integrin was increased with ageing in the vasculature of hypertensive rats (Bezie *et al*. 1998, Intengan *et al*. 1999, Intengan & Schiffrin 2000), its expression was not different from that in age-matched WKY rats (Intengan *et al*. 1999). However, no direct evidence is available on microvascular endothelial surface expression or Fg binding activity of these Fg receptors during hypertension, diabetes or stroke. Interestingly, $\alpha_5\beta_1$ integrin was strongly involved in the regulation of arterial stiffness due to its activation by higher shear stress during hypertension (Bezie *et al*. 1998). Changes in shear stress during hypertension may modulate the avidity and affinity of integrins including $\alpha_5\beta_1$ (Urbich *et al*. 2000). Thus, it is possible that surface expression and activity of endothelial ICAM-1 and $\alpha_5\beta_1$ integrin are increased during the development of cardiovascular and cerebrovascular diseases and contribute to the detrimental effects induced by increased content of Fg.

ICAM-1 and $\alpha_5\beta_1$ integrin signalling involved in endothelial cell contractility—Activation of vascular endothelial cell intraluminal surface receptors triggers many signalling cascades, some of which result in alteration of vascular reactivity. ICAM-1 ligation mediated by antibody cross-linking in brain microvascular endothelial cells, induced tyrosine phosphorylation of focal adhesion kinase (FAK) and activated c-Jun N-terminal kinase (JNK), but not ERK (Etienne *et al*. 1998). In contrast, Fg binding to endothelial ICAM-1 resulted in activation (phosphorylation) of ERK1/2 (Pluskota & D'Souza 2000, Sen *et al*. 2009). Integrin signalling involves both ERK and JNK pathways (Schlaepfer & Hunter 1998, Giancotti & Ruoslahti 1999, Hynes 1999, Shyy & Chien 2002). Activation of integrins such as $\alpha_5\beta_1$ leads to activation of multiple kinases (e.g. FAK, Src, Fyn, Shc) (Shyy & Chien 2002). Activation of FAK/Src that is associated with the actin cytoskeleton leads to JNK activation, and Fyn/Shc is linked to ERK activation (Giancotti & Ruoslahti 1999). In addition, activation of ICAM-1 induces activation of various cellular events leading to activation of members of the Rho small GTPase family, including RhoA, Cas, Crk, Cdc42 and Rac (Etienne *et al*. 1998), and results in activation of JNK (Giancotti & Ruoslahti 1999). RhoA, Cdc42 and Rac have distinct functions in regulating the actin-based cytoskeletal structure. RhoA enhances cell contractility and actin stress fiber formation, Cdc42 modulates filopodia formation, while Rac modulates membrane ruffling (Lim *et al*. 1996, Van Aelst & D'Souza-Schorey 1997). Activation of p38 mitogen-activated protein kinase (MAPK) (Buchsbaum *et al*. 2002) regulates F-actin formation (Garcia *et al*. 2002). Integrin activation is directly associated with members of the Rho family (Giancotti & Ruoslahti 1999). Fg binding ICAM-1 and possibly $\alpha_5\beta_1$ integrin, activated ERK 1/2 signalling leading to the formation of F-actin and cellular contractility (Tyagi *et al*. 2008).

ET-1 and its receptors, Fg-induced release of ET-1 and role of NO—The vascular endothelium releases agents that affect vascular tone by inducing vasodilation [prostaglandin I (PGI_2), NO and endothelium-derived hyperpolarizing factor] and vasoconstriction (thromboxane A_2 and ET-1). In addition to modulating vascular tone (Burnstock & Ralevic 1994, Yanagisawa *et al*. 1998), these agents affect platelet adhesion and aggregation (Willoughby & Loscanzo 2002). During hypertension, although total production of NO was unchanged in hypertensive compared with normal rats, bioavailability of NO was decreased (Li & Joshua 1993). Greater tissue, but not plasma ET-1, content was found in deoxycorticosterone-acetate salt-treated hypertensive rats (Zhao *et al*. 2000). As NO is an antagonist of ET-1 (Yanagisawa 1994), reduced NO bioavailability may allow ET-1 to have a pronounced effect on vascular tone during hypertension. Therefore, increased vascular ET-1 production may be one of the early events resulting in decreased resting diameters of first- and

second-order arterioles in rats with early stage of hypertension development compared with their appropriate normotensive control rats (Meininger *et al*. 1981, Harper & Bohlen 1984, Bohlen 1989). In addition, small arterioles of the rat skeletal muscle constrict more during hypertension than comparable sized vessels in normotensive rats (Bohlen 1979), suggesting that vascular reactivity is enhanced during hypertension.

Endothelins, first purified from cultured ECs by Yanagisawa *et al*. (1998), consist of three isoforms, ET-1, ET-2 and ET-3, each containing 21 amino acids (Inoue *et al*. 1989). The primary source of ET-1 is the endothelium, while ET-2 and ET-3 are present in nonvascular tissue (Russell *et al*. 1998a). ET-1, the most potent vasoconstrictor currently known, produces long-lasting arterial constriction in humans and rats (Deng *et al.* 1995). In the last two steps of its synthesis, a pre-propeptide is cleaved by a protease from big ET-1, which is cleaved by endothelin converting enzyme (ECE) to form ET-1 (Schmitz-Spanke & Schipke 2000). ET-1 is synthesized and segregated in the rough endoplasmic reticulum-Golgi system and is stored primarily in WPb (Fukushige *et al*. 2000). Activation of ECs by agonists such as thrombin, adrenaline or histamine causes exocytosis of WPbs and release of their contents into the blood circulation (Russell *et al*. 1998b). Recent data indicate that an elevated content of Fg enhances ET-1 production from cultured ECs (Sen *et al*. 2009). These results suggest that ET-1 is released from WPb through a regulatory pathway. However, there are indications that ET-1 is also discharged constitutively from the ECs (Nakamura *et al*. 1990).

The ET-A receptor (with greater affinity for ET-1) is located on VSMCs, while endothelin type B (ET-B) (a non-selective receptor) is primarily located on the ECs and VSMCs (Inoue *et al*. 1989, Deng *et al*. 1995, Russell *et al*. 1998b, Yanagisawa *et al*. 1998, Willoughby & Loscanzo 2002). ET-A and ET-B receptors regulate vascular tone in various blood vessels and vascular beds (Yanagisawa 1994) by mediating sodium and calcium ion channel function (Marsault *et al*. 1991) and by affecting ET-B, receptor-mediated release of NO and PGI_2. Most of the ET-1 secretion (~75%) from cultured ECs is towards the VSMC (abluminal) side (Yoshimoto *et al*. 1991), where it binds to ET-A receptors on the VSMCs and causes vasoconstriction. Under normal physiological conditions, ET-1 and NO are constitutively released by the endothelium and provide a balance between vascular constrictor and dilator activity (Nakamura *et al*. 1990). However, during various pathologies such as hypertension, when bioavailability of NO is decreased (Li & Joshua 1993) and regulated production of ET-1 is increased due to increased Fg content, there is a tendency towards constriction. Interestingly, an *in vivo* study of dose-dependent, ET-1-induced microvascular constriction showed that microvessels did not constrict to low doses of ET-1 (Roberts *et al*. 1998). However, if the vessels were pre-treated with N^{w}-nitro-$_{L}$-arginine methyl ester, the vascular constrictions in response to ET-1 were greatly enhanced and were significant even at low doses of ET-1 (Roberts *et al*. 1998). Because endothelin induces NO production (King *et al*. 1997, Srivastava & Magazine 1998, Ishizuka *et al*. 1999), NO may counterbalance and therefore prevent further increases in vascular constriction at higher doses of Fg.

Signalling in ET-1 production, and a role of F-actin—The role of MAPKs in ET-1-induced vasoconstriction during hypertension has been shown (Watts 2000). However, a complete understanding of the signalling mechanisms of ET-1 production remains unclear. Transcription of ET-1 depends on ERK activity (Morey *et al.* 1998). ET-1 gene expression in cultured ECs also involves the ERK pathway (Juan *et al*. 2004). Activation of ERK-1/2 is involved in Fg-induced regulated production of ET-1 (Sen *et al*. 2009). A role of the JNK-mediated pathway in production of prepro-ET-1 gene transcription has also been demonstrated (Yamakaw *et al*. 2002). However, the role of these MAPKs in ET-1 production during hypertension is not established. In several studies, activation of endothelial ICAM-1 caused activation of p38 MAP kinase and resulted in formation of F-actin through the small heat shock protein 27 (Hsp27) (Wang & Doerschuk 2001, Wang *et al*. 2005). As formation of F-actin is

required for exocytosis of WPbs (Manneville *et al*. 2003), and increased content of Fg enhances formation of F-actin (Tyagi *et al*. 2008) and production of ET-1 (Sen *et al*. 2009), it is reasonable to hypothesize that Fg binding to endothelial ICAM-1 (and possibly $\alpha_5\beta_1$ integrin) causes accumulation of WPbs in the vicinity of the EC membrane. Fg binding may also cause exocytosis of WPB through ERK (and possibly JNK and/or p38) signalling, which results in release of big ET-1 and ECE from these granules (Sen *et al*. 2009) (Fig. 2). Subsequently, formed ET-1 binds to ET-A receptors on VSMCs and causes vasoconstriction (Lominadze *et al*. 2005, Sen *et al*. 2009) (Fig. 2).

Fg and endothelial layer integrity; effect on tight junction proteins

Endothelial cells are connected to each other by a complex set of junctional proteins that comprise tight junctions, gap junctions and adherens junctions. Occludin, claudins and junctional adhesion molecules are the main tight junction proteins (TJPs) (Mehta & Malik 2006). TJPs create a paracellular barrier in endothelial cells forming a first layer of protection that separates them from blood (Förster 2008). Vascular hyperperme-ability triggered by inflammation in the heart, brain or lung promotes oedema, exacerbates disease progression and impairs recovery (Weis 2008).

Although digestion of Fg by plasmin *in vivo* is quite rare (Boutcher *et al*. 1996, Gaffney 2001), increased vascular permeability has been found to result from the interaction of thrombin and Fg (Johnson *et al*. 1983, 1985). It has been shown that the Fg degradation product, fragment D, increases endothelial layer permeability *in vitro* (Ge *et al*. 1991, 1992). Infusion of fragment D into conscious rabbits increased vascular permeability to albumin (Manwaring & Curreri 1981). However, another study showed that infusion of fragment D alone in sheep did not alter pulmonary transvascular fluid and protein exchange (Johnson *et al*. 1985). Vascular leakage was increased only if thrombin-induced pulmonary microembolization preceded fragment D infusion, suggesting a role of increased capillary hydrostatic pressure (Johnson *et al*. 1985).

A recent study by Guo *et al*. (2009) suggested that binding of the Fg-γ C terminus to endothelial $\alpha_v\beta_3$ integrin caused leakage of mesenteric microvessels through a RhoA-dependent mechanism. Another study indicated that elevated content of un-degraded Fg enhanced the formation of filamentous actin (F-actin), leading to increased endothelial layer leakage to albumin (Tyagi *et al*. 2008). In addition, leakage of Fg itself through the EC layer was also increased if its content was elevated (Tyagi *et al*. 2008), confirming the possibility that Fg leakage through the vascular wall was induced by different agonists (Pedersen *et al*. 1991) or even diseases (Areekul 1986). A role for Fg binding to endothelial ICAM-1 and $\alpha_5\beta_1$ integrin was suggested in the Fg-induced increased EC layer permeability (Tyagi *et al*. 2008). In this study, an effect of Fg-to-$\alpha_5\beta_1$ integrin binding on EC permeability, confirmed the results of Qiao *et al*. (1995), indicating that the RGD peptide increases endothelial hydraulic conductivity. These data provide an explanation for Ancrod being ineffective in treating myocardial ischaemia-reperfusion, as it reduces blood content of Fg, but at the same time increases the content of its degradation products, which may exacerbate vascular injury (Zacharowski *et al*. 2006).

Recently published data indicate that the integrity of the EC monolayer was impaired by elevated un-degraded Fg (Patibandla *et al*. 2009). A role for activation of ERK-1/2 following binding of Fg to endothelial ICAM-1 was suggested as a possible mechanism for disruption of EC layer integrity and increased permeability to albumin (Tyagi *et al*. 2008, Patibandla *et al*. 2009). In addition, increased content of Fg caused a decrease in expression of the actin-associated proteins, occludin, zonula ocluden-1 and zonula ocluden-2 (Patibandla *et al*. 2009). Fg binding to endothelial ICAM-1 was implicated in the alteration of TJPs (Patibandla *et al*. 2009) and activation of matrix metalloproteinases (MMPs) (Patibandla PK, Tyagi N, Tyagi SC, Dean WL, Roberts AM, Lominadze D, unpublished data), confirming the results

indicating that ICAM-1 is a key player in activation of MMP-9 during T lymphoma/EC interaction (Aoudjit *et al*. 1998). Taken together, these data suggest that an elevated content of Fg may increase permeability of the EC layer through formation of F-actin, downregulation of TJPs, and activation of MMPs. The activation of MMPs may cause digestion of adherens junction proteins (e.g. VE-cadherin) and this sequence of events may result in increased leakage of Fg to the sub-endothelium and its accumulation in this subcellular compartment.

Of the endothelial $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Luscinskas & Lawler 1994) that act as Fg receptors (Plow *et al.* 2000), only $\alpha_5\beta_1$ integrin has been reported to reside at the endothelial cell-to-cell contact border (Lampugnani *et al*. 1991). $\alpha_v\beta_3$ integrin was not found in intercellular contact regions (Lampugnani *et al*. 1991). Fg binds to endothelial $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins through its two RGD sequences on the Aα chain (Suehiro *et al*. 1997), and to ICAM-1 through a discrete region of the γ chain (Altieri *et al*. 1995). Therefore, depending on the Fg binding sites (ICAM-1, $\alpha_5\beta_1$ integrin, $\alpha_v\beta_3$ integrin) or their relative expression levels on the EC surface, it is possible that different physiological responses may be produced. Further studies are necessary to delineate the effects of intact Fg on vascular endothelium and SMCs, and the resultant vascular responses.

Fibrinogen-induced albumin leakage may also occur through transendothelial extravasation. Binding of Fg to ECs and activation of ERK signalling triggers albumin extravasation by caveolae via an absorptive (receptor-mediated) or fluid-phase pathway (John *et al*. 2003). Albumin can be easily taken up by caveolae (Predescu & Palade 1993). ET-1 may also have an effect on both paracellular and transendothelial transport mechanisms. However, Fg and ET-1 may utilize different mechanisms and have different potencies in inducing macromolecular leakage through the EC layer. Further experiments are needed to determine the roles and mechanism of Fg and ET-1 in macromolecular leakage through the EC monolayer.

Increases in microvascular permeability may lead to deposition of Fg in the subendothelial matrix. A strong association between elevated levels of Fg and formation of atherosclerotic plaques has been found (Bennett 2001, Lowe & Rumley 2001). Increased binding of Fg to the vascular endothelium and its leakage into the subendothelial matrix may provide favourable conditions for its conversion to fibrin by thrombin, and its generation, which is increased during hypertension (Iida *et al*. 2008) and diabetes (Cohen *et al*. 2002). Increased levels and/or activity of the plasminogen system have not been observed during a majority of the cardiovascular and cerebrovascular diseases. For example, activity of tissue plasminogen activator is diminished in brains of patients with Alzheimer's disease and mouse models of the disease (Ledesma *et al*. 2000). Thus, an increase in blood content of Fg, in the context of decreased or unaltered activity of the plasminogen system, leads to an enhanced deposition of Fg on the vascular endothelium and/or the subendothelial matrix. Immobilized Fg is then converted to fibrin by thrombin, as its degradation system can no longer counterbalance excess formation of fibrin. Enhanced deposition of fibrin exacerbates circulatory complications of cardiovascular diseases, such as the development of atherosclerotic plaques (Smith 1994), neurovascular damage and neuroinflammation (Paul *et al.* 2007). In addition, even a subtle effect of plasmin on Fg or fibrin, leading to the formation of their degradation products such as fragment D, will amplify the destructive effects initiated by an increased content of Fg.

Summary and conclusions

The data reviewed here strongly indicate that an increased Fg content may lead to significant deleterious effects in the microcirculation. As cardiovascular and cerebrovascular diseases such as hypertension, diabetes and stroke are accompanied by increased blood levels of Fg, its detrimental effects may be more pronounced during these inflammatory diseases. Binding of Fg to its endothelial receptors may activate signalling cascades that alter vasoactivity through

disruption of ET-1/NO bioavailability, increased EC layer permeability, increased formation of F-actin and disruption of the TJPs, and enhanced activity of MMPs that may digest adherens junction proteins. In addition, increased levels of Fg increase plasma viscosity and RBC aggregation, causing an increase in blood viscosity. This process may lead to an increase in blood shear stress, activation of vascular adhesion molecules and integrins, and platelets. These events may lead to even greater binding of Fg to vascular endothelium and an enhanced platelet thrombogenesis.

Lastly, the authors emphasize the potential clinical importance of increased blood Fg content. Understanding the mechanisms of destructive effects of Fg exacerbating complications of vascular dysfunction during cardiovascular diseases should allow for the development of interventions that diminish these complications.

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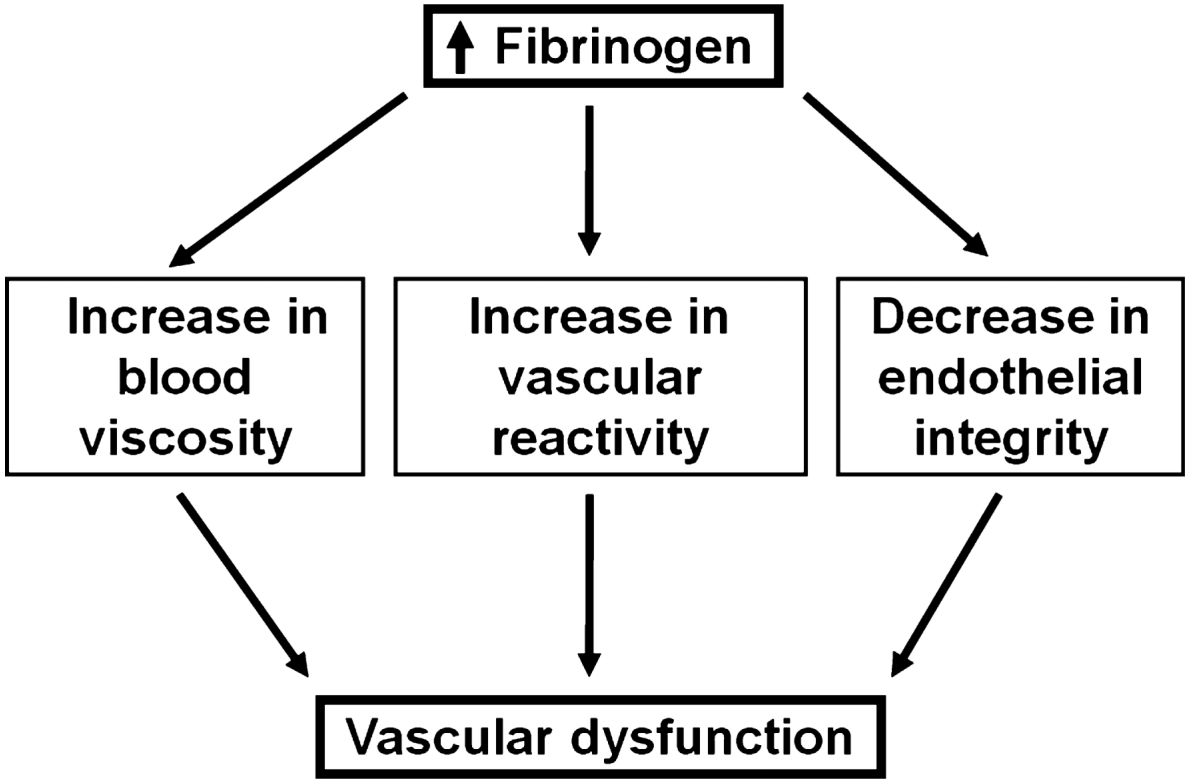

Figure 1.
Schematic representation of fibrinogen-induced vascular dysfunction.

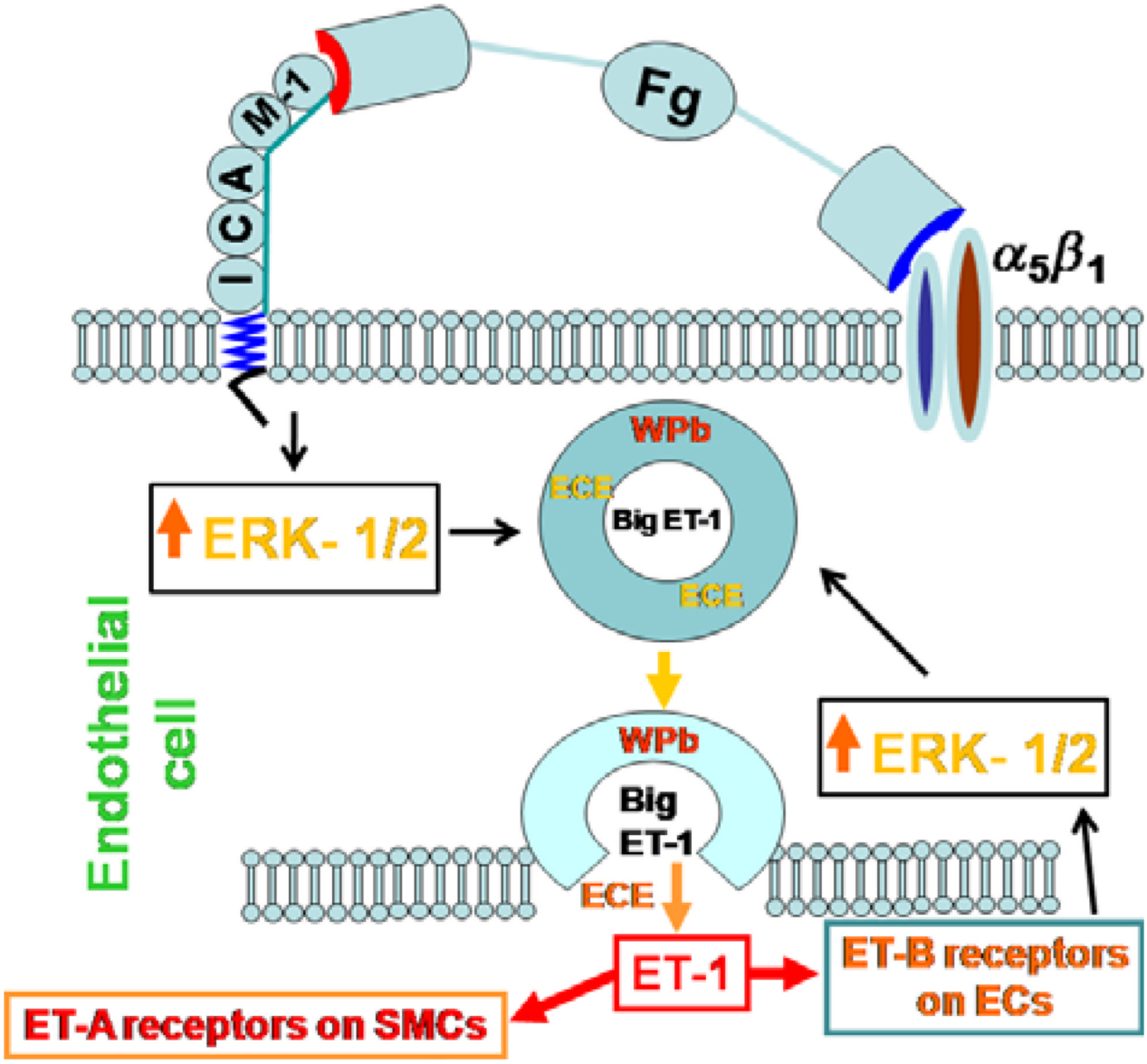

Figure 2.
Possible mechanism for fibrinogen (Fg)-induced vasoconstriction. Fg binds to endothelial intercellular adhesion molecule-1 (ICAM-1) and possibly $\alpha_5\beta_1$ integrin, induces activation of extracellular signal-regulated kinase-1/2 (ERK-1/2) signalling causing exocytosis of Weibel-Palade bodies (WPbs), formation of endothelin-1 (ET-1), and thus subsequent vasoconstriction through involvement of endothelin type A and B receptors (ET-A and ET-B respectively). Notice a negative feedback loop that involves ET-B-induced activation of ERK-1/2 signalling. Printed with permission from Sen *et al*. (2009).