RESEARCH ARTICLE

Free Fatty Acids Modulate Thrombin Mediated Fibrin Generation Resulting in Less Stable Clots

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Abstract

Upon platelet activation, free fatty acids are released at the stage of thrombus formation, but their effects on fibrin formation are largely unexplored. Our objective was to characterize the kinetic effects of fatty acids on thrombin activity, as well as the structural and mechanical properties of the resultant fibrin clots. Thrombin activity on fibrinogen was followed by turbidimetry and detailed kinetic characterization was performed using a fluorogenic short peptide substrate. The viscoelastic properties of fibrin were measured with rotatory oscillation rheometer, whereas its structure was analyzed with scanning electron microscopy (SEM). In turbidimetric assays of fibrin generation, oleate and stearate at physiologically relevant concentrations (60–600 μM) produced a bell-shaped inhibitory dose response, increasing 10- to 30-fold the time to half-maximal clotting. Oleate inhibited thrombin activity on a short peptide substrate according to a mixed-type inhibitor pattern (a 9-fold increase of the Michaelis constant, K_m and a 20% decrease of the catalytic constant), whereas stearate resulted in only a minor (15%) drop in the catalytic constant without any change in the K_m. Morphometric analysis of SEM images showed a 73% increase in the median fiber diameter in the presence of stearate and a 20% decrease in the presence of oleate. Concerning the viscoelastic parameters of the clots, storage and loss moduli, maximal viscosity and critical shear stress decreased by 32–65% in the presence of oleate or stearate, but loss tangent did not change indicating decreased rigidity, higher deformability and decreased internal resistance to shear stress. Our study provides evidence that free fatty acids (at concentrations comparable to those reported in thrombi) reduce the mechanical stability of fibrin through modulation of thrombin activity and the pattern of fibrin assembly.

Introduction

Atherothrombotic lesions of stenosed intracranial and extracranial vessels may lead to microembolization resulting in multiple small cerebral infarcts and progressive cognitive impairment [1–3]. Moreover, microembolization is reported to be the ultimate cause of

myonecrosis in patients dying of acute coronary thrombosis [4]. Peripheral occlusions in the extremities, mesenteric or renal arteries due to arterial embolism may also have serious consequences such as ulceration, gangrene, amputation or even death [5,6]. Despite the efforts made in the last two decades to explore previously unknown mechanisms of thrombus formation and to characterize the resultant clot stability (reviewed in [7,8]), our understanding of the non-conventional determinants of the structure and mechanical stability of thrombi is still far from being complete.

In addition to the release of a broad spectrum of hemostatic proteins and signal molecules during platelet activation, the increase in the cytosolic calcium concentration leads to activation of the cytosolic phospholipase-A_2. This enzyme hydrolyses membrane phospholipids releasing free fatty acids and lysophospholipid [9]. The high degree of platelet compaction in arterial thrombi [10], and the activation-dependent lipidomic flux in platelets [11], result in accumulation of phospholipids and fatty acids at concentrations in the millimolar range [12]. The major phospholipid in platelet membranes is lecithin and more than 40% of its total fatty acid content is represented by oleic (18:1) and stearic (18:0) acid [13]. Thus, the remodeling of phospholipids within thrombi raises the concentrations of oleic and stearic acid above their plasma levels (180 and 70 µM, respectively [14]). Being present both at the clotting phase and the breakdown of thrombi, free fatty acids are potential modulators of thrombus formation and thrombolysis. The presence of fatty acids in the clot is known to directly affect the activity of proteases involved in fibrin degradation [12,15,16], but their effects on the enzyme activities in fibrin formation (e.g. thrombin) and the resultant clot structure and stability have hardly been investigated.

Variations in fatty acid content at the level of the whole body are known to affect the procoagulant mechanisms. Recent reports evidence reduced thrombin generation after consumption of fatty acids [17–19]. Inhibition of thrombin by oleic and stearic acid has been reported in amidolytic assays [20]. Furthermore, fatty acid esters of polyphenols are more efficient inhibitors of thrombin, than free polyphenols [21]. Hindrance of thrombin activity occurs mostly through noncovalent interactions, as demonstrated for several natural [22–24] and synthetic [25] thrombin inhibitors, however feasible molecular interactions between free fatty acids and thrombin as well as the exact kinetic characteristics of thrombin-mediated clot formation in the presence of these modulators are scarcely explored. The possibility of a noncovalent interaction between free fatty acids and fibrinogen has also emerged, since molecules forming hydrogen bonds with fibrinogen, may alter the structure of the fibrin clots due to the major role of H-bonds in the polymerization step of fibrin clot formation [26].

The overall aim of this study was to characterize the kinetic effects of free fatty acids on thrombin activity and also the structural and mechanical properties of the formed clots. Our study revealed that free oleic and stearic acids at biologically relevant concentrations modify the kinetics of fibrin formation and the ultrastructure of the fibrin network, resulting in clots that can be mechanically disassembled at shear stress of magnitude corresponding to the hydrodynamic conditions in partially occluded coronaries [27]. The impaired mechanical stability of fibrin suggests that the free fatty acid content of arterial thrombi [12] could contribute to the risk of microembolization *in vivo*.

Materials and Methods

Proteins and reagents

If not otherwise indicated, experiments were performed in HEPES buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, pH 7.4) using bovine thrombin, purchased form Serva Electrophoresis GmbH (Heidelberg, Germany) and further purified as described in [28] yielding a preparation with specific activity of 2100 IU/mg [29]. Thrombin activity of 1 IU/mL was

considered equivalent to approximately 10.7 nM by active site titration [30]. Human thrombin (01/580 the WHO 2nd International Standard for alpha thrombin) was obtained from NIBSC (South Mimms, UK). Sodium salt of oleic and stearic acids were from Sigma-Aldrich Kft. (Budapest, Hungary) and stock solutions (10 mM) were prepared in water (prewarmed to 70˚C) containing 50 µM butylated hydroxytoluene. These stock solutions were further diluted to the desired concentrations in HBS. (At the final concentration in the reaction mixtures butylated hydroxytoluene had no effect on the thrombin activity on its own). Fibrinogen (human, plasminogen-depleted) was from Calbiochem (San Diego, CA, USA) and fluorogenic thrombin substrate butoxycarbonyl-Val-Pro-Arg-7-amido-4-methylcoumarin (Boc-VPR-AMC) was from R&D Systems (Minneapolis, MN, USA).

Kinetics of fibrin formation by thrombin

Turbidimetric assays were performed to investigate the effect of free fatty acids on the kinetics of fibrinogen clotting by thrombin. Fibrinogen at 7.5 µM was clotted with 20 nM thrombin in the presence of 20–800 µM sodium oleate or 25–1500 µM sodium stearate in microplate wells at 37˚C. The course of clot formation was monitored by measuring the light attenuation at 340 nm with a Zenyth 200rt microplate spectrophotometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) and T_{50} values (the time needed to reach the half maximal turbidity) were determined.

Kinetics of short peptide hydrolysis by thrombin

Because turbidimetric measurements generate a combined signal that reflects the outcome of fibrinogen cleavage by thrombin and the polymerization of fibrin monomers, an alternative fluorometric assay on the substrate Boc-VPR-AMC was used to directly characterize the effect of free fatty acids on the catalytic activity of thrombin. Following preliminary estimates of the Michaelis constant (K_m) for each modulator concentration, hydrolysis of Boc-VPR-AMC (at six different concentrations ranging from $0.5K_m$ to $5K_m$) by 10 nM thrombin was monitored in the presence of 0; 50; 100 or 200 µM sodium oleate or stearate at 37˚C. In some cases 100 µM bovine serum albumin was added to the reaction mixture as a positive control. Fluorescence intensity (which reflects the release of amido-methylcoumarin) was measured continuously for 80 s with a CLARIOstar® microplate reader (BMG LABTECH GmbH, Ortenberg, Germany) (excitation: 380 nm, emission: 460 nm). The delay time between the initiation of the reaction and the first measurement point was estimated with linear extrapolation from the initial six measured RFU (Relative Fluorescence Units) values back to baseline fluorescence, and a coefficient of 262.7 RFU µM^{-1} cm^{-1} (determined from calibration in our assay system) was used to convert the measured fluorescence values to product concentration. The kinetic parameters of thrombin were estimated according to the following model mechanism

$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\longrightarrow} E + P$, where E is thrombin, S is Boc-VPR-AMC, P is amido-methylcoumarin and k_1, k_2 and k_{-1} are the respective reaction rate constants. With the quasi-steady-state assumption the differential rate equation for this scheme is

$$\frac{dP}{dt} = \frac{k_p . E_{t0} . (S_0 - P)}{K_m + S_0 - P}, \qquad \text{(Eq. 1)}$$

where E_{t0} and S_0 are the initial concentrations of thrombin and its substrate, the Michaelis constant $K_m = (k_{-1}+k_2)/k_1$ and the catalytic constant $k_p = k_2$ [15].

A previously described numerical procedure [15] including generation of 1000 synthetic sample sets for each experimental setting and Monte Carlo simulation of the reaction progress

curves was applied to identify the final best estimates and the 95% 'root' confidence intervals of the catalytic constant (k_p) and K_m according to the integrated form of Eq 1:

$$ t = \frac{1}{k_p.E_{t0}} P + \frac{K_m}{k_p.E_{t0}} \ln \frac{S_0}{S_0 - P} \, . \tag{Eq. 2} $$

The abovementioned numerical evaluation comprises a table look-up procedure instead of the regression analysis of the linearized version of Eq 2. All calculations were performed in Matlab R2016a (The MathWorks, Inc., Natick, MA, US).

Rheological Measurements of Fibrin Clots

The effects of free fatty acids on the viscoelastic properties of fibrin clots were studied in a cone-and-plate type oscillation rheometer (HAAKE RheoStress 1, Thermo Scientific, Karlsruhe, Germany). Fibrinogen at 6 μM, premixed with 100 μM sodium oleate or stearate was clotted with 10 nM thrombin in the measurement gap of the rheometer at 37˚C. An oscillatory strain (1 Hz, 0.015 strain amplitude) was imposed on the samples, and viscoelastic parameters (storage modulus, *G'* and loss modulus, *G"*) were recorded with the help of the HAAKE RheoWin v. 3.50.0012 data manager software (Thermo Scientific) for 10 min, a time interval sufficient to establish the trend of the measured parameters in all samples. For determining the gel/fluid transition of the same clots, a stepwise increasing shear stress (τ) of 0.01 to 1000 Pa was applied and dynamic viscosity (η) was determined at each step. For numerical description of flow curves, maximal viscosity (η_{max}) and critical shear stress values (τ_{flow}, at which viscosity falls to zero and gel/fluid transition occurs) were determined. The statistical significance of differences in the parameters was evaluated with the Kolmogorov-Smirnov test at $p < 0.05$ level.

Scanning Electron Microscope Imaging of Fibrin Clots

To examine the effect of free fatty acids on the structure of fibrin, 7.5 μM fibrinogen was clotted with 20 nM thrombin for 3 hours at 37˚C in the presence of 40–400 μM sodium oleate or 50–500 μM sodium stearate. The fibrin clots were fixed in 1%(v/v) glutaraldehyde in 100 mM sodium cacodylate, pH 7.2, dehydrated in a series of ethanol dilutions, ethanol/acetone and pure acetone followed by critical point drying with CO_2 in an E3000 Critical Point Drying Apparatus (Quorum Technologies, Newhaven, UK). The specimens were mounted on adhesive carbon discs, sputter-coated with gold in an SC7620 Sputter Coater (Quorum Technologies), and images were taken with a scanning electron microscope (SEM) EVO40 (Carl Zeiss GmbH, Oberkochen, Germany). Fibrin fiber diameters were measured and data distributions were analyzed using Kuiper's test and Monte Carlo simulation procedures running under Matlab R2016a [31,32].

Particle Size Determination by Dynamic Light Scattering

The particle size distribution profile of the sodium oleate and stearate solutions used in the above-mentioned experiments (0; 200; 400; 800 or 1200 μM sodium oleate or stearate in the presence of 7.5 μM fibrinogen in HBS) was measured with a W130i dynamic light scattering system (AvidNano, London, UK). Measurement data analysis including determination of the polydispersity index (PDI) was performed with the i-Size software, supplied with the system by the manufacturer. PDI is the square of the light scattering polydispersity (the ratio of absolute width and mean value of the size distribution) of dissolved particles. A PDI value of 0 refers to a monodisperse system with uniform particles, while a PDI of >0.4 suggests a polydisperse system with particle sizes varying too widely for exact size discrimination.

SDS Polyacrylamide Gel Electrophoresis of Thrombin Autodigestion Products

To investigate the impact of free fatty acids on the process of thrombin autocleavage, as an indicator of molecular interactions between the enzyme and the modulators, human thrombin at 6 μM was incubated with 0 or 1 mM of sodium oleate or stearate at 37°C for 72 hours. Samples were treated with non-reducing denaturing buffer (50 mM Tris-HCl, 50 mM NaCl, 2% (w/v) SDS, pH 8.2) and heated at 95°C for 3 min. Gel electrophoresis was performed on a 10–15% (w/v) gradient polyacrylamide gel, followed by visualization of protein bands with silver staining.

Results

Effects of free fatty acids on thrombin activity

Two different kinetic assays were applied to investigate the influence of free fatty acids on thrombin activity. A turbidimetric assay was performed to monitor the action of thrombin on its natural substrate, fibrinogen in the presence of sodium salts of fatty acids. The increase of absorbance in this assay reflects the formation of polymerizing fibrin, the final structure of which causes variations in the absolute maximal values of absorbance. In this assay the time to reach half-maximal turbidity (T_{50}) can be used to describe the kinetics of fibrin formation, because its values do not depend on these structural variations of the final clots. We found that both oleate and stearate delayed the clotting process and produced a bell-shaped inhibitory dose response of thrombin-mediated fibrin generation. Stearate had a stronger impact on the rate of clot formation: T_{50} increased 38-fold at the most effective modulator concentration (300 μM), while oleate caused up to a 12-fold increase in the T_{50} values (Fig 1).

In the second kinetic assay, thrombin activity was measured on a fluorogenic peptide substrate, which allowed a detailed analysis of the kinetic parameters (k_p, K_m) of thrombin in the presence of fatty acids. In this experimental setup stearate was found to have no biologically relevant effect on the kinetic parameters, whereas oleate exhibited a mixed-type inhibitory
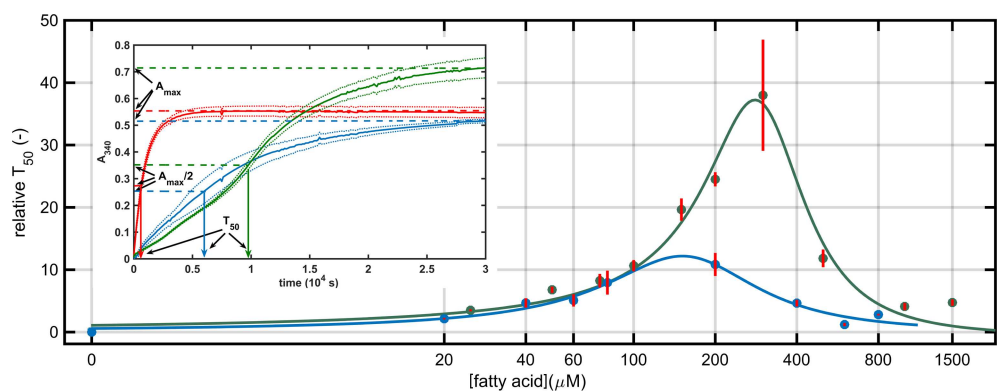

Fig 1. Effect of free fatty acids on the kinetics of thrombin-mediated fibrin generation. Fibrinogen was clotted with thrombin in the presence of sodium oleate or stearate. The course of clot formation was monitored by measuring the absorbance at 340 nm and T_{50} was defined as the time needed to reach the half-maximal turbidity (illustrated in the Inset for clotting in the absence of any additive in red, in the presence of 200 μM stearate in green or oleate in blue as mean with continuous lines ± 1SEM with dotted lines, $n = 5$). Relative T_{50} values are presented in green for stearate and blue for oleate (T_{50} measured in the absence of additives is considered to be 1) as mean (symbols) and SEM (red bars), n = 5. Lines represent the optimal fit to a ratio of empirical polynomial functions with a degree of 2 for the power coefficient in both the numerator and denominator functions (Curve Fitting Tool 3.5.3 of Matlab 2016a).
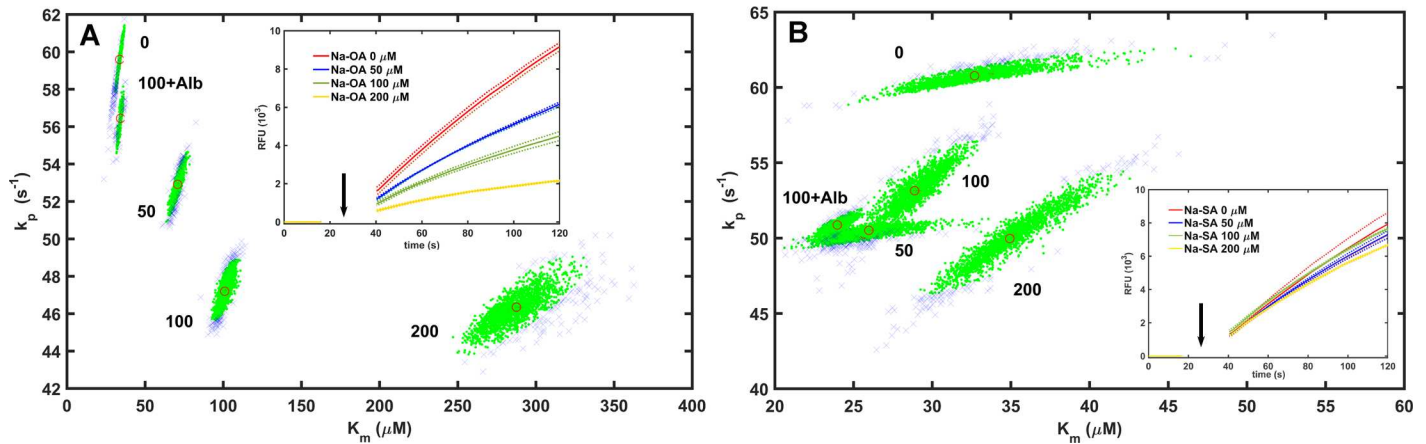
Fig 2. Effect of free fatty acids on the kinetic parameters of thrombin on a small peptide- substrate. Boc-VPR-AMC at 6 different concentrations adjusted to match the range of $0.5K_m$ to $5K_m$ for each modulator concentration was added to thrombin and different concentrations of sodium oleate (A) or stearate (B) (three parallel series of each substrate concentrations were measured for each fatty acid concentration). The release of amido-methylcoumarin was measured continuously and the progress curves were analyzed to estimate the kinetic parameters (k_p, K_m) according to the procedures described in the Materials and methods. The estimated synthetic parameter pairs within the 95% confidence region are shown by green symbols and the pairs out of this region are presented in blue (the numbers indicate the concentration of the fatty acid in µM, for which the respective data set was generated in the Monte-Carlo simulation, +Alb indicates the presence of 100 µM albumin). The best estimate from the Monte-Carlo simulation is indicated by a red circle. The exact numerical values are presented in Table 1. Insets show progress curves (mean with continuous lines ± 1SEM with dotted lines, $n = 3$) at 80 µM Boc-VPR-AMC and different sodium stearate or oleate concentrations (the arrows indicate the initiation of the reaction with thrombin injection into the substrate).

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effect on the enzyme, decreasing k_p from 59.67 (57.85–61.41) to 46.23 (43.83–49.04) s^{-1} and increasing K_m from 33.88 (31.07–36.84) to 285.80 (246.98–332.42) µM (best estimates and their 95% 'root' confidence intervals) (Fig 2, Table 1). Equimolar concentrations of albumin that binds the free fatty acids completely abrogate the inhibiting effect of oleate (Table 1).

To address the mechanism of these inhibiting effects, the autodigestion of thrombin was examined in the presence and absence of fatty acids. In the absence of additives gel electrophoresis did not detect any autodigestion during the first 72 hours, whereas oleate and stearate both accelerated the self-destroying enzymatic process but with a different size profile of the degradation products (Fig 3). Differences in the band sizes in the case of oleate and stearate are attributable to ongoing autocleavage of presumably different mechanisms.

Table 1. Kinetic parameters of thrombin in the presence of free fatty acids.

	oleate (µM)						stearate (µM)					
	0		**50**	**100**		**200**	**0**		**50**	**100**		**200**
		+Alb			**+Alb**			**+Alb**			**+Alb**	
K_m (µM)	**33.9**	**45.6**	**70.8**	**100.8**	**33.9**	**285.8**	**32.5**	**32.0**	**25.5**	**28.9**	**24.0**	**34.9**
95% 'root' CI	31.1–36.8	43.3–48.2	63.5–78.6	92.6–111.0	31.7–36.2	246.9–332.4	24.7–46.4	30.0–34.4	20.6–33.6	25.0–32.9	22.0–25.9	29.3–42.6
k_p (s^{-1})	**59.7**	**66.1**	**52.9**	**47.2**	**56.4**	**46.2**	**60.7**	**68.3**	**50.3**	**53.2**	**50.9**	**50.0**
95% 'root' CI	57.9–61.4	64.7–67.5	50.9–54.7	45.7–48.9	54.6–58.1	43.8–49.0	58.9–62.6	66.7–69.7	49.3–51.3	50.1–56.4	49.8–51.9	46.3–54.4

Numerical values for the Michaelis constant (K_m) and the catalytic constant (k_p) of thrombin on Boc-VPR-AMC substrate were determined as illustrated in Fig 2 and are presented as best estimates and their confidence intervals (CI) from Monte-Carlo simulation of 1000 cycles performed as described in Materials and methods. The presence of 100 µM albumin is indicated as +Alb.

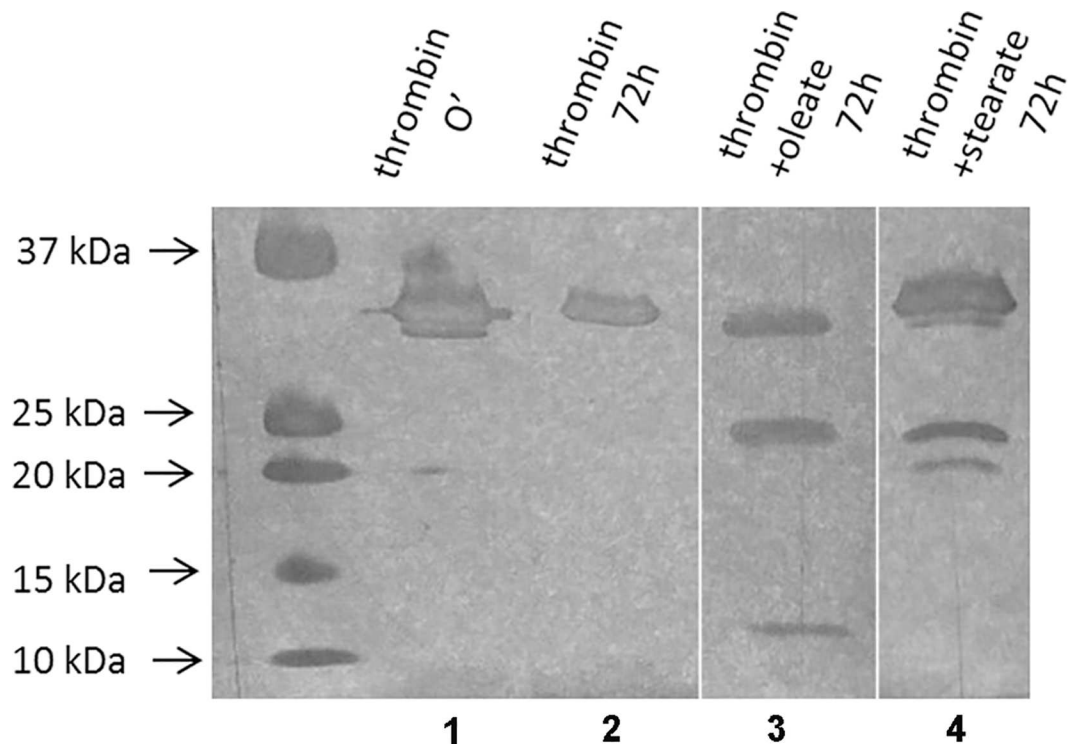doi:10.1371/journal.pone.0167806.t001

Fig 3. Effect of free fatty acids on thrombin autodigestion. Thrombin was incubated in the presence or absence of sodium oleate or stearate at 37˚C for 72 hours. Samples were treated with a non-reducing denaturing buffer and polyacrylamide gel electrophoresis was performed, followed by visualization of protein bands by silver staining. Samples run on different gels are separated by vertical white lines. Lanes 1 and 2: Thrombin incubated in the absence of modulators for 0 min and 72 hours. Lanes 3 and 4: Thrombin incubated in the presence of oleate or stearate for 72 hours.

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Because in our experimental setup fatty acids are exposed to pH below their pK_a values (10.15 for stearic- and 9.85 for oleic acid) [33], most probably they participate in the form of aggregates as suggested by the results of the dynamic light scattering measurements evidencing extreme polydispersity (PDI>2) for fibrinogen-fatty acid-HBS solutions. Exact particle size determination could not be performed, since PDI values over 0.4 refer to an extremely broad size distribution. Sodium salts of fatty acids give a transparent, colorless solution when dissolved in water containing butylated hydroxytoluene at 37˚C. However, when diluted in HBS at pH 7.4, their solution turns opalescent indicating aggregate or microcrystal formation. The opalescence is more intense in the case of stearate in line with its melting point of 70˚C, much higher than that of oleic acid (13–14˚C).

Effects of free fatty acids on the structural and mechanical properties of fibrin clots

Because fibrin turnover *in vivo* depends on the lytic susceptibility and mechanical stability of the clots and these properties are directly affected by the three-dimensional structure and viscoelastic characteristics of fibrin [28,31,34,35] and reviewed in [36, 37], it was of interest to investigate the impact of fatty acids on the structure and mechanical stability of fibrin. Morphometric analysis of SEM images showed significant fiber thickening at 100–200 μM of stearate, with a maximum increase of the median diameter by 73% (from 50.5 to 87.3 nm), while fiber diameter values approximated control levels at 500 μM stearate yielding a bell-shaped
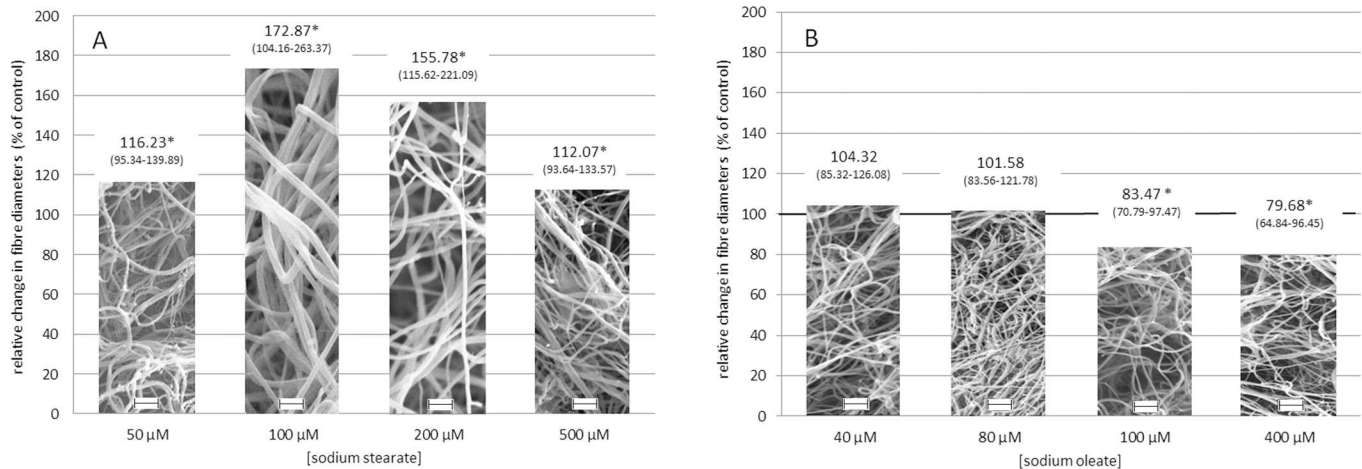
Fig 4. Effect of free fatty acids on fibrin fiber diameters. Fibrinogen premixed with sodium stearate (A) or oleate (B) at the indicated concentrations was clotted with thrombin. Clots were processed for SEM imaging and fibrin fiber diameters were measured and analyzed as detailed in Materials and methods. Bars contain representative images of fibrin with the indicated additive (scale bar = 1 µm) and the median values (bottom—top quartiles) of the diameter distributions are shown above each bar. The height of the bars indicates the relative change in fiber diameter as percentage of the median values in the absence of additives. Asterisk indicates statistical significance at p<0.001 according to Kuiper's test in comparison to pure fibrin.

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dose-dependence, similar to the dose-response curve in Fig 1. Oleate had a weak thinning effect producing a 20% drop in median fiber diameter at 400 µM oleate (Fig 4).

The viscoelastic properties of the clots were determined by oscillatory rheometry. In the course of fibrin polymerization, the storage (G') and loss (G") moduli of the samples increased over time (Fig 5). In line with the turbidity data, the rheometry measurements evidenced slower clotting and lower values of both storage and loss moduli in the presence of fatty acids, while the loss tangent (G"/G') remained unchanged (Table 2). When increasing shear stress was imposed upon completely polymerized clots, critical shear stress values (τ_{flow}) and maximal viscosity (η) decreased by 32–65% at 100 µM fatty acid concentrations as shown in Table 2 and Fig 6.

Discussion

Despite many advances in diagnosis and management, complications of arterial embolism are still a leading cause of disability and death. Most arterial emboli originate in the left heart, manifesting mainly in embolic stroke or transient ischemic attack. The second most common cause of morbidity and mortality from arterial embolic disease is limb ischemia in the lower extremities. Less frequently, emboli target the upper extremities, mesenteric or renal arteries [38]. Embolization may be a consequence of mechanical instability of the fibrin clot in the subacute phase of an arterial occlusion or as a response to the thrombolytic or invasive endovascular intervention [39–41]. Thus, during the treatment of arterial thrombosis at different locations (acute ischemic stroke, myocardial infarction or peripheral arterial occlusion) the possibility of embolization has to be considered as a crucial determinant of the therapeutic outcome. The mechanical stability of an early thrombus is the overall consequence of the presence and actual local concentration of the molecular and cellular participants of clot formation. Therefore, different genetic, environmental or acute pathophysiological factors may lead to altered clot structure and stability [7,8,28,31]. During the process of arterial thrombus formation, free fatty acids are released from activated platelets and therefore emerge as potential
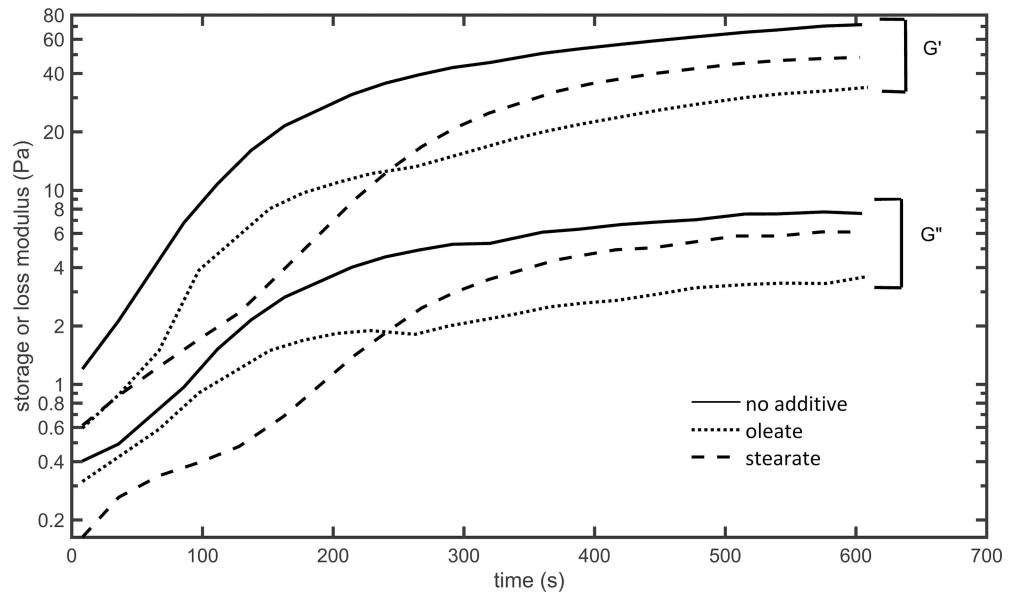
Fig 5. Effect of free fatty acids on the course of rigidity changes during clot formation. Fibrinogen premixed with sodium oleate or stearate was clotted with thrombin in the measurement gap of a cone-and-plate type oscillation rheometer and an oscillatory strain was imposed on the samples. Representative curves of storage (G') and loss (G'') moduli for each clot type are presented, all rheological parameters with statistics are shown in Table 2.

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modulators of fibrin clot formation. Our present study addressed the impact of free fatty acids on thrombin mediated fibrin generation and the mechanical properties of the formed clot.

The bell shape of the response in clotting time to the applied dose of fatty acid (Fig 1) suggests a physical interaction between thrombin and the fatty acid in the form of a ternary complex, in which fibrinogen or fibrin monomer also participates. In this ternary complex, the modulator fatty acid exerts a kinetic template effect by promoting the favorable spatial organization for the inhibition of the enzyme reaction or modification of the polymerization process. Maximal alteration occurs at an optimal concentration ratio of the three components [42]. An analogous ternary complex has been reported previously consisting of thrombin, fibrin monomer and heparin, the formation of which altered the activity of thrombin on its different substrates [43]. Due to the known resistance of thrombin to autolysis [44], the kinetics of thrombin autodigestion is rather slow to account for the observed inhibition of clotting, though its acceleration in the presence of fatty acids suggests molecular interactions between fatty acids and thrombin even in the absence of fibrin(ogen). This physical interaction affects the action of thrombin on low-molecular weight peptide substrates, too. Both fatty acids

Table 2. Effect of free fatty acids on the viscoelasticity of fibrin clots.

	G' (Pa)	G'' (Pa)	loss tangent	η (kPa·s)	τ_{flow} (Pa)
no additive	73.1 (1.6)	7.9 (0.2)	0.1 (0.004)	10.9 (0.8)	522 (46)
oleate	36.7* (4.5)	3.8* (0.5)	0.1 (0.001)	3.9* (1.1)	353* (31)
stearate	46.5* (7.5)	5.4* (0.8)	0.1 (0.003)	3.8* (1.5)	346* (74)

Mean values for storage modulus (G'), loss modulus (G''), their ratio (G''/G', loss tangent) after 10 min clotting, maximal viscosity values (η) and critical shear stress (τ_{flow}) values are presented (with standard deviations).

* Asterisk indicates $p < 0.05$ according to the Kolmogorov-Smirnov test in comparison to pure fibrin, $n = 4$.
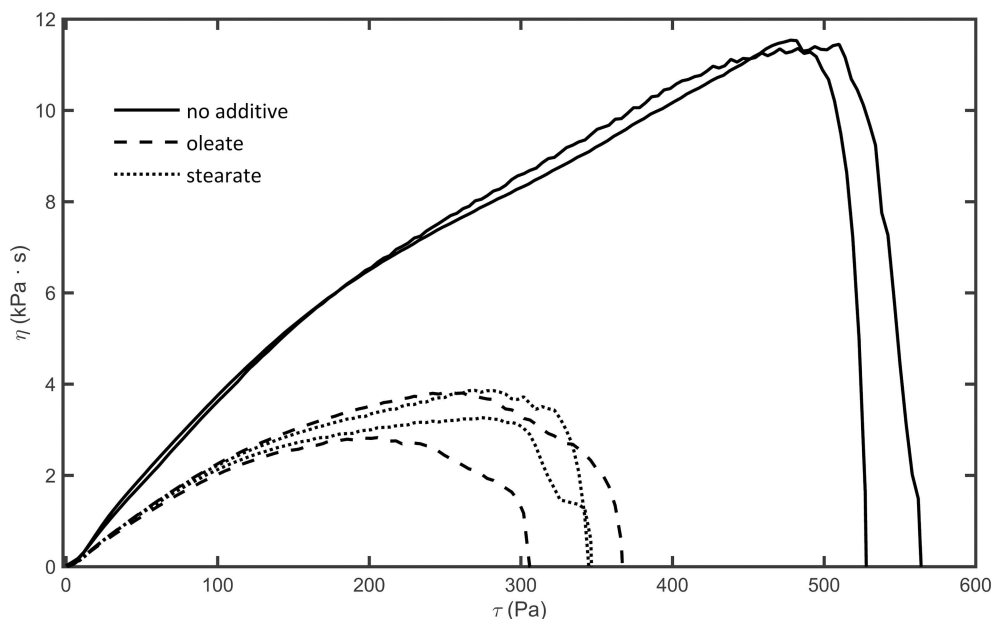
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Fig 6. Effect of free fatty acids on the gel/fluid transition of fibrin clots. Fibrin clots were prepared in the measurement gap of a cone-and-plate type oscillation rheometer as detailed in Materials and methods. Thereafter, stepwise increasing shear stress (τ) was applied to the clot and dynamic viscosity (η) was determined. The abrupt fall in viscosity to 0 indicates the gel/fluid transition of the fibrin clots (τ_{flow} in Table 2). Two representative curves are shown for each clot type.

altered the thrombin activity on Boc-VPR-AMC, but with different kinetic patterns: 200 μM oleate caused a marked, 8-fold increase in K_m, while both fatty acids resulted in a minor drop in the catalytic constant.

Dynamic light scattering data indicate free fatty acids form aggregates under our experimental conditions and aggregated long chain fatty acids are likely to form hydrogen bonds with fibrinogen and thrombin in the proposed ternary complex through carboxylic groups. Hydrophobic interactions between a few alkyl chains (which may be available on the surface of the fatty acid aggregate) and hydrophobic regions of fibrinogen and the active site and/or Exosite I of thrombin are also conceivable [45,46]. Differences in the effects of stearic acid and oleic acid could be traced back to known differences in their aggregate assembly. Because of the kink in the aliphatic chain at the *cis*-double bond, oleic acid forms loose aggregates with 50% greater intermolecular distance between the polar groups on their surface than stearic acid [33], which could also result in a different steric assembly of the ternary complex.

The ultimate structure and mechanical properties of fibrin depend on the relative kinetics of two sequential steps in its formation: 1) catalytic action of thrombin on fibrinogen, and 2) polymerization of the formed fibrin monomers. Each of these steps could be independently targeted by modulators of fibrin formation. In most cases a decrease in thrombin activity leads to a coarser fibrin network with less frequently branched thicker fibers consisting of more protofibrils [34], but incorporation of non-fibrin molecules into the fibrin meshwork modifies the fiber diameter independently of the thrombin concentration, e.g. DNA increases [31], whereas heparin decreases fiber size [47] without any direct effect on thrombin activity. The marked prolongations in clotting times, as well as the major structural-mechanical changes in the presence of both stearate and oleate are clearly disproportionate with the minor changes in the k_p values in our enzyme kinetic measurements using a small synthetic peptide substrate. Further discrepancy is the stearate increasing fibrin fiber diameters, which could alone easily be

explained by lower thrombin activities and prolonged clotting times, however we found no fibrin fiber thickening in the presence of oleate, despite a marked increase in K_m for the fluorogenic substrate and a delay in clotting. Thus, such disproportionate structural changes might be attributed mainly to alterations in the polymerization step of fibrin formation and to a lesser extent to effects through thrombin activity. The maximal prolongation of clotting times by the fatty acids could be explained by a subtle delay in fibrin polymerization due to the presence of fibrin(ogen)-thrombin-fatty acid ternary complexes, and potentially fibrinogen-fatty acid aggregates, which -in the case of stearate- could incorporate into fibrin bundles, and appear as fibrin fiber thickening. A similar phenomenon has been reported by Yeromonahos et al., observing increased fibrin porosity and fiber diameters due to incorporation of thrombin-fibrin monomer-unfractionated heparin ternary complexes into the fibers [48].

Despite the differences in fiber diameters, the mechanical properties of the clots formed in the presence of the two fatty acids were rather similar according to the viscoelastic parameters gained by rheometry. A mechanically less stable fibrin network is suggested by the diminished values of the storage and loss moduli (G' and G"). The loss tangent (G"/G'), however, remained unchanged. Data in the literature [49] suggest that the formation of thicker fibers consisting of more protofibrils due to lower thrombin activity result in increased clot rigidity (G'). Moreover, since these clots usually also present with less frequent branching, these thicker fibers tend to suffer a higher degree of intrafiber rearrangement upon deformation, which results in a higher energy loss observed as a higher loss tangent value [49]. The rheological effects of fatty acids in our experiments are commensurate with a different mechanism, which also argues indirectly against the possibility of fatty acids inhibiting thrombin activity on fibrinogen. Thinner fibers, higher clot deformability (decreased G' and G") and an unchanged loss tangent have been described in fibrin structures formed at decreasing fibrinogen concentrations [49], which suggests that the presence of fatty acids may result in less effectively polymerizing fibrin monomers. In the case of oleate, thinner fibers were formed (similar to the effect observed at a lower fibrinogen concentration), while incorporation of the abovementioned ternary complexes overcomes this effect when stearate is present during the clotting phase. The dynamic viscosity (η_{max}) of fibrin was also lowered in the presence of fatty acids indicating decreased internal resistance. Moreover, according to the τ_{flow} values, less energy was necessary to reach the gel-fluid transition, the point, at which the fibrin matrix lost its physical integrity. Thus, mechanically less stable and more deformable clots were formed, when free fatty acids were present at physiological concentrations during fibrin polymerization. If we compare the shear stress values reported for partially occluded blood vessels (about 230 Pa at 80% occlusion of coronary arteries) [27] and the shear stress needed to reach the gel-fluid transition in our experimental setup, it can be concluded that stearic and oleic acid decrease the critical shear stress necessary for disassembly of fibrin to values that are likely to act on *in vivo* thrombi.

Conclusions and Perspectives

Since fibrin structure is a major determinant of the mechanical stability and lytic susceptibility of thrombi [36,50], we can conclude that free fatty acids at their physiological concentrations may destabilize intravascular thrombi. These *in vitro* findings prompt further investigations on the free fatty acid content of embolizing *ex vivo* thrombi so that predictive conclusions for prevention of *in vivo* microembolization could be drawn in the clinical practice.

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