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Inhibition of plasmin-mediated prostromelysin-1 activation by interaction of long chain unsaturated fatty acids with kringle 5

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Abstract

C18 unsaturated fatty acids were here found to inhibit proMMP (matrix metalloproteinase)-3 activation by plasmin. This effect was suppressed by lysine ligand competitors, indicating that it was mediated by binding to kringle domains. Surface plasmon resonance analysis demonstrated that oleic acid interacted to a similar extent with plasmin and kringle 5 (K_D values of 3.4×10^{-8} and 5.9×10^{-8} M) while interaction with kringles 1–2–3 was 10-fold lower. Furthermore, oleic acid stimulated the amidolytic activity of plasmin and mini-plasmin, but not micro-plasmin. Oleic acid also enhanced u-PA (urokinase-type plasminogen activator)-mediated plasminogen activation over 50-fold. Taken together, these data indicate that inhibition of plasmin-induced proMMP-3 activation by unsaturated fatty acids was mediated through their preferential binding to kringle 5. The influence of elaidic acid on the plasmin/MMP-3/MMP-1 proteolytic cascade was assessed *ex vivo*. Exogenous addition of plasmin to dermal fibroblasts or supplementation of gingival fibroblast culture medium with plasminogen triggered this cascade. In both instances, elaidic acid totally abolished proMMP-3 and proMMP-1 activation. Additionally, a significant decrease in lattice retraction and collagen degradation in a range similar to that obtained with Batimastat was observed when human gingival fibroblasts were cultured in plasminogen-containing type I collagen gels, indicative of the dual influence of unsaturated fatty acids on MMP activation and activity. In conclusion, unsaturated fatty acids or molecules with similar structures could be attractive target for the development of natural pharmacological inhibitors directed against plasmin and/or MMPs in different pathological contexts such, skin UV irradiation, vascular diseases and tumour growth and invasion.

Keywords: Matrix metalloproteinases; Stromelysin-1; Plasmin; Fatty acids; Kringles; Human fibroblasts

1. Introduction

MMPs form a family of zinc-dependent metallo-endopeptidases involved in the degradation of various ECM components [1,2]. They play an essential role in tissue remodelling and in various physiological and pathological conditions such as angiogenesis, morphogenesis, rheumatoid arthritis, osteoarthritis, tumour progression, and cardiovascular diseases [3–6]. MMPs are synthesised by a variety of cell types and most of them are secreted from cells as latent forms (proMMPs). While activation of proMMPs can occur via mechanisms such as oxidation

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Abbreviations: MMPs, matrix metalloproteinases; ECM, extracellular matrix; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; TIMPs, tissue inhibitor of metalloproteinases; LBS, lysine binding sites; FnII, fibronectin type II; 6-AHA, 6-aminohexanoic acid; AMCHA, tranexamic acid; K1, kringle 1; K5, kringle 5; SA, stearic acid; OA, oleic acid; EA, elaidic acid; cPAR, *cis*-parinaric acid; tPAR, *trans*-parinaric acid; LLA, linoleic acid; LNA, linolenic acid; SPR, surface plasmon resonance; BSA, bovine serum albumin; FCS, foetal calf serum; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris buffer saline.

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and nitrosylation [7], activation is primarily brought about by the action of proteolytic cascades, mainly catalysed by neutral proteinases [8]. Plasminogen and stromelysin-1 (MMP-3) have been shown to play pivotal role in these cascades [9–11]. MMP-3 is a member of the stromelysin subfamily of MMPs that displays broad specificity towards a variety of ECM components [1,2], and also participates directly or indirectly in several MMP activation cascades [9,12–14]. These properties make MMP-3 an attractive target for inhibitor design and pharmacological development. Plasmin efficiently activates prostromelysin-1 (proMMP-3) and both enzymes co-operate to activate the zymogen form of collagenase-1 (proMMP-1) [9]. The activation of plasminogen to plasmin is mediated by tissue-type plasminogen activator (t-PA) or by u-PA [15,16]. The interaction of u-PA with its high affinity cellular receptor (u-PAR) leads to a rapid generation of plasmin [15,17,18]. Individual components of the plasmin and MMP-3 proteolytic systems act together in the pericellular environment to initiate focal proteolysis [19]. The presence of such a cascade has been investigated *ex vivo* by He *et al.* [20] using organ culture and, more recently by Berton *et al.* [21] using type I collagen lattices populated with gingival fibroblasts.

Physiological control of the plasmin/MMP proteolytic cascade is exerted at several distinct levels including gene expression, plasminogen and MMP activation and enzyme inhibition by plasminogen activator inhibitors (PAIs) and TIMPs [22–24]. This control regulates both cell adhesion to the ECM and cell migration through tissue boundaries. In addition to TIMPs and PAIs, other organic molecules have been reported to modulate the plasminogen activation system and the proteolytic activities of plasmin and MMPs. We recently reported that the catalytic activities of gelatinases A and B (MMP-2 and MMP-9, respectively) and MMP-1, on both synthetic and protein substrates, were inhibited by unsaturated fatty acids [25,26]. A minimal chain length of 18 carbon atoms and the presence of at least one double bond were found to be essential for enzyme inhibition. These unsaturated fatty acids such as oleic acid, inhibited gelatinases more strongly than MMP-1, due to their preferential binding to the FnII-like domains of the gelatinases, which are not present in MMP-1 [27]. Higazi *et al.* [28] have also reported that unsaturated fatty acids inhibit the u-PA-mediated and plasminogen-dependent cleavage of fibrin clots. They also showed that oleic acid could modulate plasmin activity [29]. Such an effect appeared to be mediated by the LBS located within the five kringle (K1–K5) domains of plasmin(ogen). Those domains are involved in specific interactions with fibrin and more generally in the regulation of plasmin activity [30–36]. Lysine or structural analogues such as 6-AHA or AMCHA can bind to kringle domains [37]. The gelatin binding site of the first module of the FnII repeats in MMP-2 contains identical topological features to the LBS of kringle domains in plasmin(ogen) [31,38].

Since native Glu-plasminogen as well as Lys-plasminogen were also recently found to bind to MMP-3 with high affinity [39], we therefore hypothesised that fatty acids might interfere with plasmin-mediated proMMP-3 activation. Here we demonstrated that the preferential interaction between long-chain unsaturated fatty acids and kringle 5 of human plasmin inhibits proMMP-3 activation both *in vitro* and *ex vivo* using dermal or gingival fibroblasts cultures expressing different levels of plasminogen activators [40,41]. Experiments performed with gingival fibroblast-populated type I collagen lattices support the conclusion that long chain unsaturated fatty acids are important regulators of tissue proteolysis by virtue of their dual inhibition of plasmin-mediated proMMP-3 activation and MMP activity.

2. Experimental procedures

2.1. Reagents

Analytical grade chemicals were purchased from Prolabo unless otherwise specified. Human plasmin (6-AHA, lysine free), human Glu-plasminogen (6-AHA, lysine free), u-PA and truncated (hemopexin deleted) MMP-3 were purchased from Calbiochem represented by VWR International. Miniplasmin (consisting of K5 and the proteinase domain of plasmin) and microplasmin (consisting of the proteinase domain) were generous gifts of Prof. R. Lijnen (Centre for Molecular and Vascular Biology, University of Leuven, Belgium). Human proMMP-3 and proMMP-1 were obtained from Valbiotech. Recombinant K5 of human plasminogen, expressed in *Escherichia coli* as previously described [42], was a generous gift of Prof. Y. Cao (Laboratory of Angiogenesis Research, Karolinska Institute, Stockholm, Sweden). The free acid forms of octadecanoic acid (SA, C18:0), *cis*-9-octadecenoic acid (OA, C18:1Δc9), *trans*-9-octadecenoic acid (EA, C18:1Δt9), *cis*-9,*cis*-12-octadecadienoic acid (LLA, C18:2Δc9,c12) and *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (LNA, C18:3Δc9,c12,c15), BSA, phorbol myristate acetate (PMA), aprotinin from bovine lung (affinity purified), benzamidine, gelatine, 6-AHA, AMCHA, LBS-I corresponding to kringles 1–3 of plasminogen, BCIP/NBT and anti-mouse IgG peroxidase conjugated polyclonal antibodies were purchased from Sigma. The free acid forms of *cis*-9,*trans*-11,*trans*-13,*cis*-15-octadecatetraenoic acid (cPAR, C18:4Δc9,t11,t13,c15) and *trans*-9,*trans*-11,*trans*-13,*trans*-13-octadecatetraenoic acid (tPAR, C18:4Δt9,t11,t13,t15) were obtained from Molecular Probes. Batimastat, a synthetic MMP inhibitor, was generously provided by British Biotechnology. Monoclonal antibodies to human MMP-3 and MMP-1 were from Oncogene. Monoclonal antibody to human urokinase (MAB 1337) was purchased from Chemicon. The chemiluminescent enhanced kit was from Amersham Biosciences Europe.

The synthetic chromogenic substrates S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide) and S-2444 (pyro-Glu-Gly-Arg-*p*-nitroanilide) were from Chromogenix. The fluorescent quenched substrate Mca-PLGL-Dpa-AR-NH_2 was obtained from Bachem. Plastic tissue culture dishes were from Nunc. Tissue culture media, including FCS, were from Gibco. Acid-soluble collagen from rat tail was prepared according to a previously described method [43].

2.2. proMMP-3 activation

Human plasmin (65 nM) was preincubated in the absence or presence of fatty acids (50 μM) or aprotinin (50 μM) in 50 mM Tris–HCl buffer, pH 7.8, containing 150 mM NaCl and 5 mM $CaCl_2$ for 15 min at 37°. proMMP-3 (100 nM) was then added and the reaction was allowed to proceed for 4 hr at 37°. The experiments were performed in the presence of 10 μM Batimastat to abolish the proteolytic action of generated MMP-3. At the end of the incubation aprotinin (0.3 mg/mL) was added to inactivate plasmin. In some experiments, 6-AHA or AMCHA were added during the preincubation period. The activation of proMMP-3 by plasmin was then evaluated by Western blot analysis.

2.3. SDS–PAGE and Western blot

MMP-1 and MMP-3 were analysed by Western blotting. Samples were submitted to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% (w/v) acrylamide under non-reducing conditions according to Laemmli [44] and proteins were electroblotted to PVDF membranes (Immobilon-P from Millipore) [45]. The membranes were blocked with 5% (w/v) non-fat dry milk (Biorad) in 50 mM TBS buffer, pH 7.5, containing 150 mM NaCl for 2 hr at 20°. The blotted proteins were probed with primary antibody diluted in TBS-Tween (0.1% v/v) (TBS-T) containing 1% non-fat dry milk (w/v) for 5 hr at 20°. Monoclonal antibodies against human MMP-1 and MMP-3 were used at 1/5000. After extensive washings with TBS-T, the membranes were incubated with peroxidase-conjugated goat anti-mouse IgG (1/10,000) for 1 hr at 20°. After extensive washings with TBS-T, the immunoreactive proteins were revealed by staining with enhanced chemiluminescent detection reagents or with NBT/BCIP reagent.

2.4. Real-time biomolecular interaction analysis of plasmino(gen) binding to fatty acids

The inter-actions of OA, EA and SA with plasmin and plasminogen were studied using a BIAcore X system (Pharmacia Biotech). Sensor chips HPA (BIAcore AB) were used for all experiments. A flow rate of 5 μL/min was used and experiments were performed at 25°. Fatty acids were coated onto the sensor chip by spontaneous adsorption. The surface of sensor chip was washed with 40 mM *n*-octyl-β-D-glucopyranoside in water for 5 min. Fatty acids [20 μL of a 1 mM solution in 50% (v/v) dimethyl sulfoxide (DMSO)] were then injected and adsorption monitored. Additional injections were performed until the sensorgram reading reached a stable level. The formation of multiple lipid layers on the sensor chip surface was eliminated by increasing the flow rate to 100 μL/min for 5 min and by an additional injection of 10 mM NaOH (10 μL). To assess the extent of coverage of the surface, 10 μL of 0.1 mg/mL BSA in 0.1 M Tris–HCl buffer, pH 7.8 was injected. The amounts of BSA bound to the sensor chip surface corresponded to 43 resonance units (RU). Uncoated octylglucopyranoside-washed chips bound an average of 1000 RU. Plasmin(ogen), LBS-I, and K5 binding experiments were also performed in 0.1 M Tris–HCl buffer, pH 7.8 at different concentrations as indicated in the text. After each cycle, the sensor chip was regenerated by injection of 10 mM HCl/glycine, pH 1.1 to restore the baseline to the level observed before injection. The effect of various lysine ligand analogues on plasmin binding was investigated by their inclusion at several concentrations in the buffer during the association phase at a fixed concentration of plasmin (640 nM). Their competitive effect was calculated from the SPR signal observed at the start of the dissociation phase deduced from the bulk contribution. The percentage of inhibition was evaluated relative to the SPR signal obtained in the absence of competitor. Equilibrium dissociation constants were determined as described in the BIAtechnology handbook (Amersham Pharmacia Biotech). Briefly, at steady state:

$$\frac{dR}{dt} = k_{ass} \times C(R_{max} - R_{eq}) - k_{diss} \times R_{eq} = 0 \quad (1)$$

which may be rearranged as follows:

$$R_{eq} = \frac{C \times R_{max}}{K_D + C} \quad (2)$$

where $K_D = k_{diss}/k_{ass}$ is the equilibrium constant, R_{eq} is the response value at steady state, R_{max} is the maximal capacity of the sensor chip for binding analyte, and C is the molar concentration of analyte. K_D was calculated by non-linear regression analysis by fitting the (R_{eq}, C) pairs to Eq. (2) using GraphPad software.

2.5. Plasmin and u-PA assays

Plasmin activity was monitored using the chromogenic substrate S-0251 as described above and according to Friberger *et al.* [46]. Briefly, 200 μL of 0.1 M Tris–HCl buffer, pH 7.8, containing 12 nM plasmin and 0.3 mM S-2251 were added in 96-well low binding titration plate (Nunc) and incubated in the presence or absence of fatty acids (0–100 μM) for various periods at 22 or 37°. The influence of fatty acids on the amidolytic activity of miniplasmin and microplasmin were compared to that

exerted on plasmin using identical amounts of active enzymes, plasmin unit is defined as the amount of enzyme that will hydrolyse 1 μmol of H-D-Val-Leu-Lys-*p*-nitroanilide. Urokinase activity was determined using S-2444 chromogenic substrate as previously described [47].

2.6. Determination of plasminogen activation by u-PA

Activation of plasminogen by u-PA was studied using a solid-phase assay. Monoclonal antibody to human u-PA was coated at various concentrations in a 96-well microtitration plate (Cliniplate EB from Labsystem) in 25 mM carbonate buffer, pH 9.6, for 18 hr at 4°. The plate was washed with 20 mM Tris–HCl buffer, pH 7.6, containing 0.15 M NaCl and 300 μL per well of 1% (w/v) BSA in the same buffer were added to block the plate (1 hr at 22°). After extensive washing in 0.15 M NaCl, 20 mM Tris–HCl buffer, pH 7.6, 100 μL per well of human uPA were added at various concentrations and incubated for 1.5 hr at 22°. Then, the plate was extensively washed in 0.15 M NaCl, 20 mM Tris–HCl buffer, pH 7.6 and 200 μL of 0.3 mM S-2251 in 0.1 M Tris–HCl buffer, pH 7.8, were added in each well in the presence or absence of plasminogen at increasing concentrations. The kinetics of plasmin generation were monitored at 405 nm and 22°. The influence of fatty acids on the activation of plasminogen by u-PA was studied in this system. For this purpose, fatty acids dissolved in DMSO were diluted in 0.1 M Tris–HCl buffer, pH 7.8, so that the final concentration of DMSO never exceeded 0.1% (v/v). In some experiments 6-AHA or benzamidine were added at 10 mM concentration.

2.7. Determination of matrix metalloproteinase activity

The influence of C18 fatty acids either saturated or unsaturated on recombinant MMP-3 activity was determined using the fluorescent quenched substrate Mca-PLG-Dpa-AR-NH_2 [27]. MMP-3 (400 pM) was mixed with 0–40 μM fatty acids in a 50 mM Hepes buffer, pH 7.5, containing 150 mM NaCl and 5 mM $CaCl_2$. The assays were initiated by adding 2 μM substrate. The reaction was allowed to proceed for 20 min at 22° and then stopped by adding 10 mM EDTA. The rate of substrate cleavage was measured in triplicate for each fatty acid concentration examined, using a Perking Elmer LS50B spectrofluorometer with excitation and emission wavelengths of 325 and 387 nm, respectively. Less than 5% of the substrate was hydrolysed during the rate measurements. K_i values were determined by plotting v_i/v_o as a function of fatty acid concentrations and non-linear regression analysis using the Grafit computer program (R. Leatherbarrow, Erithacus Software) and the integrated equation:

$$\frac{v_i}{v_o} = \frac{2[E]_0 - ([E]_0 + [I]_0 + K_i) - (([E]_0[I]_0 + K_i)^2 - 4[E]_0[I]_0)^{1/2}}{2[E]_0}$$

where v_i is the rate of substrate hydrolysis in the presence of inhibitor, v_o is the rate in its absence and $[E]_0$ and $[I]_0$ are the concentrations of enzyme and inhibitor at the beginning of the reaction, respectively [48].

2.8. Monolayer cell cultures on plastic surface

Human dermal fibroblasts obtained from a healthy subject of 35 years old and were used at the 6th passage. The informed consent of the donors was obtained, according to the declaration of Helsinki. Cells were cultured to confluence in 24-well culture plates in DMEM containing 10% (v/v) FCS at 37° in a humidified atmosphere containing 5% CO_2. Cells were incubated in FCS-free DMEM in the presence or absence of 2.5 μg/mL plasmin and in the presence or absence of 50 μM EA for 6 hr at 37°. MMPs secreted into the cell culture medium were analysed by Western blot. Gingival biopsies were obtained from patients (20–50 years old) exempt of periodontal disease, during preprosthetic surgery. The informed consent of the donors was obtained, according to the declaration of Helsinki. Human gingival fibroblasts were grown from explants in DMEM supplemented with 10% FCS, and cultured at 37° in a humidified atmosphere containing 5% CO_2. Fibroblasts at the 4th to 7th passage were cultured to confluence in DMEM supplemented with 10% (v/v) FCS in 24-wells culture plates. Fibroblasts (1,50,000 cells/well) were rinsed twice with DMEM and then, fresh medium containing 50 μM fatty acids, i.e. SA and EA were added in the absence of FCS. Plasminogen (2.5 or 15 μg/well) was added after 30 min incubation. Cells were then incubated for periods up to 48 hr. The generation of active MMP-1 and MMP-3 was followed by Western blot analysis.

2.9. Cell cultures in three-dimensional collagen lattices

Human gingival fibroblasts were used at the 4th to 7th passage. Three-dimensional lattice cultures were performed as previously described [21]. Lattices were incubated in the presence or absence of plasminogen (50 μg per lattice) and in the presence or absence of fatty acids (50 μM). The lattice diameter and the degradation of collagen lattice as determined by measurement of released hydroxyproline [49] were measured daily after seeding.

3. Results

3.1. Fatty acids inhibit plasmin activation of proMMP-3 by binding to kringle domains

The previously reported preferential binding of long chain unsaturated fatty acids to FnII like domains of MMP-2 [27], together with the structural analogy between such MMP regions and kringle domains [31,38], prompted

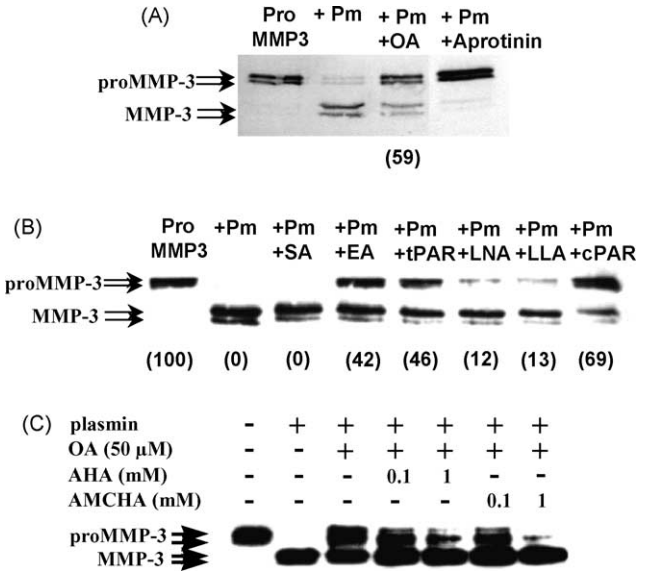

Fig. 1. Involvement of kringle domains in the fatty acids inhibition of plasmin-mediated proMMP-3 activation. (A) Human plasmin (65 nM) was preincubated for 15 min at 37° in the absence or presence of OA (50 μM) or aprotinin (50 μM) in a 50 mM Tris–HCl buffer, pH 7.8, 150 mM NaCl, 5 mM $CaCl_2$ and Batimastat (10 μM). proMMP-3 (100 nM) was then added. The reaction was stopped after 6 hr incubation at 37° by adding aprotinin (50 μM) and analysed by Western blot using polyclonal antibodies recognising both proMMP-3 and MMP-3. The immunoreactive proteins were revealed by staining with NBT/BCIP reagent. (B) The influence of SA, EA, tPAR, cPAR, LLA and LNA on the activation of proMMP-3 by plasmin were evaluated under the same conditions. Values in brackets correspond to the percentages of plasmin-mediated proMMP-3 activation by fatty acids, calculated by densitometric scanning using a Vilber Lourmat photoimager as follows: proMMP-3 × 100/(proMMP-3 + MMP-3). (C) Plasmin (65 nM), was preincubated in the absence or presence of AHA or AMCHA for 15 min and then OA (50 μM) was added for the next 15 min at 37° in 50 mM Tris–HCl buffer, pH 7.8, 150 mM NaCl, 5 mM $CaCl_2$. proMMP-3 (100 nM) was further added for 6 hr at 37° and samples were analysed by Western blot using a monoclonal antibody (clone AB-3 from Oncogene). The immunoreactive bands were revealed with anti-mouse IgG peroxidase-conjugated polyclonal antibodies and enhanced chemiluminescent detection reagents.

us to investigate the influence of these substances on plasmin-mediated proMMP-3 activation. In the absence of plasmin, two immunoreactive proMMP-3 bands were detected corresponding to the glycosylated and non-glycosylated form of proMMP-3 (Fig. 1A). Upon addition of plasmin, proMMP-3 was activated to mature MMP-3 (Fig. 1A). This activation was abolished by 50 μM aprotinin. Western blots of proMMP-3 preincubated with 50 μM OA showed that MMP-3 activation was inhibited by 59% (Fig. 1A). At the same concentration, cPAR inhibited more than OA, while EA and tPAR exhibited similar inhibitory activity (Fig. 1B). LLA and LNA were much less efficient and SA had no significant effect (Fig. 1B). Since kringle domains have been suggested to mediate the interaction of plasmin with fatty acids [28], we examined the importance of such domains in the activation of proMMP-3 by plasmin using lysine ligand analogues as competitors. Western blot analysis showed that the inhibitory effect of OA on plasmin-mediated proMMP-3 activation involved LBS since 6-AHA and particularly AMCHA substantially reduced the effect of OA (Fig. 1C). Similar results were obtained with EA instead of OA (not shown).

3.2. Preferential binding of oleic acid to kringle 5 of human plasmin(ogen)

In order to confirm the interaction of fatty acids with kringle domains of plasmin(ogen), SPR analysis was performed. Binding of various concentrations of plasminogen, plasmin, LBS-I and K5 domain to OA is shown in Fig. 2. Analysis of the association and dissociation rate constants from sensorgrams revealed a 16-fold higher OA affinity for plasminogen as compared to plasmin (Table 1). In addition, such interaction seemed to be mediated preferentially by K5, which exhibited a 10-fold higher affinity for OA than LBS-I. The interaction between plasmin and OA was found to be lysine binding site-dependent since, at a fixed concentration of plasmin, addition of increasing concentrations of 6-AHA or AMCHA completely abolished the interaction between plasmin and OA as shown by the decrease in SPR signal (Fig. 3). 6-AHA or AMCHA exhibited IC_{50} values of 188 and 45 μM, respectively. Benzamidine in the mM range also inhibited the binding of plasmin to OA (data not shown).

Fatty acids were previously reported to increase the amidolytic activity of plasmin through interaction with kringle domains of plasmin [29]. We confirmed that OA enhances the amidolytic activity of human plasmin on S-2251 substrate in a concentration dependent manner (data not shown). We also show that EA, LLA and LNA, but not SA, exert a similar effect. In order to further assess the involvement of K5 in the interaction between fatty acids and plasmin, we studied the influence of OA on

Table 1
Kinetic constants for interactions of oleic acid with plasminogen, plasmin, LBS-I or kringle 5 as determined by SPR. The sensorgrams were analysed by non-linear regression to obtain the dissociation (k_{diss}) and associatin (k_{ass}) rate constants. The dissociation constants (K_D) was obtained from the ratio k_{diss}/k_{ass}. Data represented the mean ± SD of three experiments

	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_D (M)
Plasminogen	$6.6 \pm 1.2 \times 10^{3}$	$3.7 \pm 0.6 \times 10^{-5}$	$5.6 \pm 1.2 \times 10^{-9}$
Plasmin	$4.5 \pm 1.0 \times 10^{4}$	$15.5 \pm 1.2 \times 10^{-4}$	$3.4 \pm 0.8 \times 10^{-8}$
LBS-I	$1.7 \pm 0.4 \times 10^{3}$	$9.7 \pm 0.3 \times 10^{-4}$	$5.7 \pm 1.0 \times 10^{-7}$
Kringle 5	$3.4 \pm 0.4 \times 10^{3}$	$2.0 \pm 0.3 \times 10^{-4}$	$5.9 \pm 0.9 \times 10^{-8}$

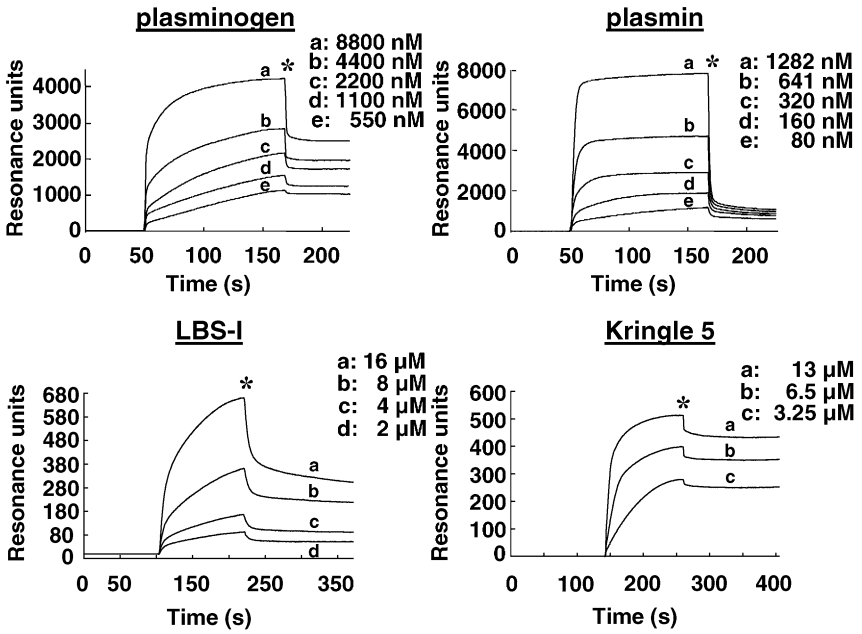

Fig. 2. Plasminogen, plasmin, LBS-I and kringle 5 binding to oleic acid by real-time biomolecular interaction analysis. Solutions of plasminogen, plasmin, lysine binding sites-I (LBS-I) and recombinant K5 in 0.1 M Tris–HCl buffer, pH 7.8, at varying concentrations were injected and allowed to bind to OA immobilized onto a HPA chip at a flow rate of 5 µL/min and at 25°. Interactions were examined by SPR analysis as described Section 2. The asterisk indicates the end of the association phase.

the amidolytic activity of plasmin, miniplasmin and microplasmin. As shown in Fig. 4A–C, the rates of S-2251 hydrolysis by plasmin, by miniplasmin devoid of K1–K4 domains, but not by microplasmin devoid of all kringle domains, were increased by OA. We then examined the influence of OA on the activation of proMMP-3 by plasmin, miniplasmin and microplasmin. Western blot analysis shows that the activation of proMMP-3 by plasmin or miniplasmin but not by microplasmin was inhibited in the presence of OA, thus confirming that K5 plays a major role in the interaction between fatty acids and plasmin (Fig. 4D).

3.3. Fatty acids enhance u-PA-mediated plasminogen activation

Although we cannot excluded a possible role for kringle 4, data obtained by SPR analysis support the hypothesis that fatty acids bind preferentially to K5, and induce a conformational change in native plasminogen that might

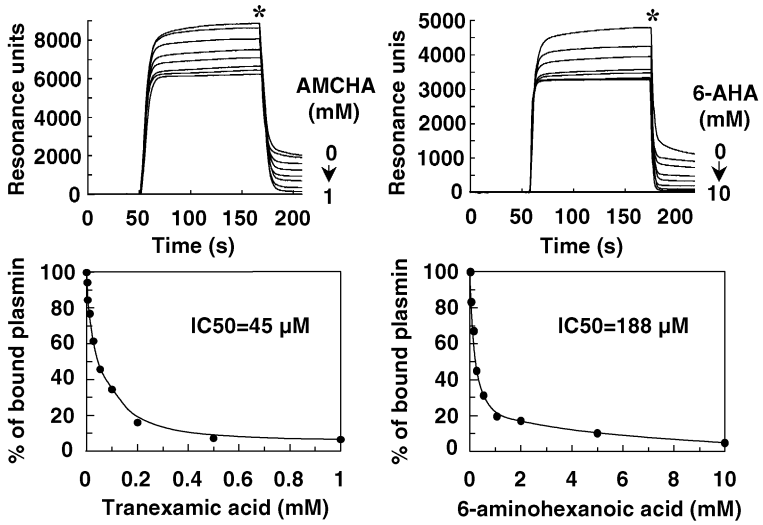

Fig. 3. AMCHA and 6-AHA compete for binding of plasmin to oleic acid. Plasmin (640 nM) as injected in the absence or presence of varying concentrations of AMCHA (0, 2.5, 10, 25, 50, 100, 200 and 1000 µM) or 6-AHA (0, 50, 100, 250, 500, 1000, 2000, 5000 and 10,000 µM) in 0.1 M Tris–HCl buffer, pH 7.8, onto an OA-covered HPA chip and interactions were examined by SPR analysis as described in Section 2. The asterisk indicates the end of the association phase. The amount of bound plasmin was evaluated from maximum resonance values obtained at the start of the dissociation phase (*) deduced from the resonance value corresponding to the bulk refractive index. The percentage of inhibition was then evaluated relative to the SPR signal obtained in the absence of competitor.

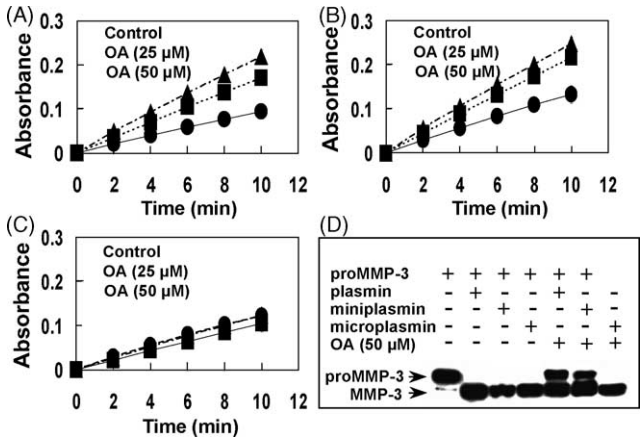

Fig. 4. Influence of OA on the amidolytic activity and proMMP-3 processing activity of plasmin, miniplasmin and microplasmin. (A, B, C) Amidolytic activity: plasmin (12 nM, 1.17 μU) was incubated in 0.1 M Tris–HCl buffer, pH 7.8, containing S-2251 (0.3 mM) in the absence (●) or presence of oleic acid 25 μM (■), 50 μM (▲) for 10 min at 22°. Hydrolysis of substrate was monitored at 405 nm. The amidolytic activity of miniplasmin (1.56 μU) (B) and microplasmin (1.89 μU) (C) were also evaluated in the presence or absence of OA under the same conditions as described above. (D) proMMP-3 activation: plasmin (9.02 μU), miniplasmin (10.04 μU) and microplasmin (9.95 μU) were preincubated in the presence or absence of 50 μM OA for 15 min at 37° in 50 mM Tris–HCl buffer, pH 7.8, 150 mM NaCl, 5 mM $CaCl_2$. proMMP-3 (100 nM) was further added for 6 hr at 37° and samples were analysed by Western blot using a monoclonal antibody (clone AB-3 from Oncogene) recognising proMMP-3 and MMP-3. The immunoreactive bands were revealed with anti-mouse IgG peroxidase-conjugated polyclonal antibodies and enhanced chemiluminescent detection reagents.

render it more susceptible to u-PA activation. We first demonstrated that EA or OA binding to Glu-plasminogen *in vitro* generated low but detectable plasmin amidolytic activity using S-2251 as substrate (not shown). In a solid-phase assay system, C18 fatty acids were found to accelerate the u-PA-mediated plasminogen activation in a concentration-dependent manner (Fig. 5). However, the potency of fatty acids to enhance plasminogen activation was neither proportional to the number of unsaturated bonds nor dependent on the *cis*/*trans* configuration of fatty acids (OA ≥ EA > SA > tPAR > cPAR). Under our experimental conditions, the lowest concentration of OA or EA still able to stimulate the activation process was 1 μM. Fatty acids had no effect on u-PA amidolytic activity.

3.4. Influence of EA on proMMP-3 and proMMP-1 activation by plasmin in human fibroblast cultures

We explored the influence of fatty acids on the plasmin-MMP-3/MMP-1 proteolytic cascade *ex vivo* using dermal fibroblast cell cultures. Culture medium was supplemented with EA, instead of OA, since EA is more stable and has only minimal effect on MMPs and TIMPs expression (data not shown). Dermal fibroblasts express negligible levels of plasminogen activators under basal conditions; so addition of plasmin (2.5 μg/mL) to the culture medium was necessary to induce the MMP-3/MMP-1 proteolytic cascades in

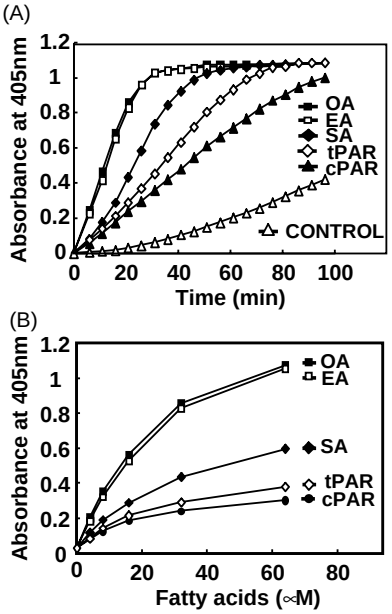

Fig. 5. Influence of C18 fatty acids on u-PA-mediated plasminogen activation. Plasminogen (0.1 μM) was incubated in 0.1 M Tris–HCl buffer, pH 7.8, containing S-2251 (0.3 mM), in the presence or absence of OA, EA, SA, cPAR or tPAR onto u-PA-coated plate at 22°. Plasmin activity was monitored at 405 nm for varying periods at fixed 32 μM fatty acid concentration (A) and varying concentrations of fatty acids for 21 min (B).

this system. This activation was significantly inhibited by EA (Fig. 6). Contrary to their dermal counterpart, gingival fibroblasts express plasminogen activators under basal conditions [40,41]. In this cell model, supplementation of cell culture medium with 15 μg/mL plasminogen (but not 2.5 μg/mL) led to generation of plasmin amidolytic activity which was significantly enhanced by EA (50 μM) (Fig. 7A). However, the induction of MMP-3/MMP-1 proteolytic cascade triggered by plasminogen addition, could be totally suppressed by EA (Fig. 7B).

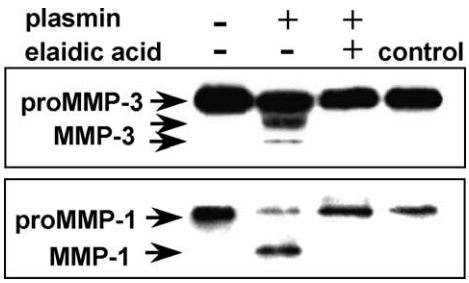

Fig. 6. EA inhibits the activation of proMMP-3 and proMMP-1 by plasmin in human dermal fibroblast cultures. Human dermal fibroblasts (6th passage) were seeded at 1,00,000 cells/well in a 24-wells plastic culture plate and cultured to confluency in DMEM containing 10% (v/v) FCS. Cells were washed three times in PBS and incubated in 0.5 mL FCS-free DMEM in the presence or absence of plasmin (2.5 μg/mL) and in the presence or absence of 50 μM EA. After 24 hr incubation at 37°, the medium was analysed for MMP-3 and MMP-1 by Western blot. The reactive bands were revealed with IgG peroxidase conjugated antibodies and enhanced chemiluminescent detection reagents. proMMP-3 and proMMP-1 controls are shown.

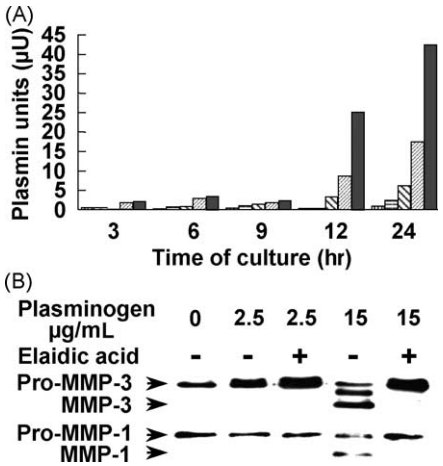

Fig. 7. Influence of EA on plasmin amidolytic activity and proMMPs activation in plasminogen supplemented human gingival fibroblast cultures. Human gingival fibroblasts (1,40,000 cells/well) were seeded in 24 wells plastic culture dishes and cultured to confluency in DMEM containing 10% FCS. Cells were then incubated in FCS-free DMEM (▥) supplemented with 2.5 μg/mL plasminogen (▤), 2.5 μg/mL plasminogen + EA (50 μM) (▧), 15 μg/mL plasminogen (▨), 15 μg/mL plasminogen + EA (50 μM) (■). (A) Plasmin amidolytic activity (μU) was determined as a function of time of culture (from 3 to 24 hr) from aliquots of culture medium using S-2251 as substrate. (B) proMMP-3 and proMMP-1 activation was assessed by Western blot at 24 hr culture.

3.5. Influence of EA on the retraction and degradation of collagen lattices populated with human gingival fibroblasts

Retraction of type I collagen lattices populated with fibroblasts is a widely used *ex vivo* model for the physiological induction of MMP proteolytic cascades, leading to conspicuous collagen degradation [21,50,51]. As depicted above, EA inhibits activation of MMP-3/MMP-1 by plasmin in two-dimensional fibroblasts culture. Additionally, this fatty acid was reported to inhibit MMP-1 and MMP-2 activity on both synthetic and natural substrates [27]. Figure 8 shows that EA, as well as other unsaturated C18 fatty acids, was also able to inhibit MMP-3 activity

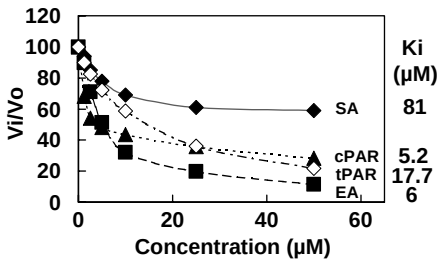

Fig. 8. Inhibition of MMP-3 activity by unsaturated fatty acids. APMA-activated hemopexin-truncated MMP-3 (400 pM) was incubated in 50 mM Hepes buffer, pH 7.5 with 150 mM NaCl and 5 mM $CaCl_2$, containing Mca-PLG-Dpa-AR in the presence or absence increasing concentrations of SA, EA, cPAR and tPAR for 20 min at 22°. Hydrolysis of substrate was monitored with excitation and emission wavelengths of 325 and 387 nm, respectively and v_i/v_o were plotted against fatty acid concentration and K_i's values are indicated in brackets.

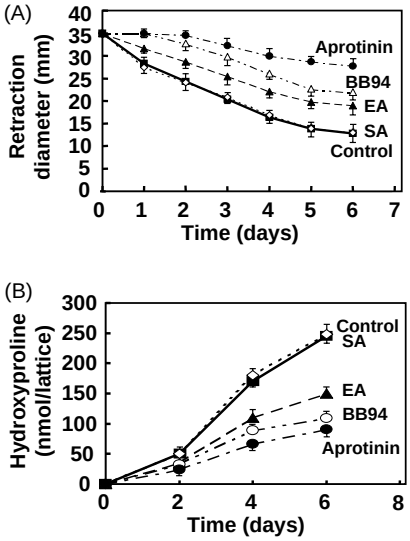

Fig. 9. Influence of EA on the retraction and degradation of gingival fibroblast-populated collagen lattices. Three-dimensional lattice cultures consisting of rat tail acid-soluble collagen (1 mg per lattice) populated with 2,00,000 gingival fibroblasts in 1 mL of DMEM containing 1% (v/v) FCS. Before cell seeding and lattice formation, plasminogen (50 μg per lattice) was added to the culture medium in the absence (control) or presence of 50 μM EA or SA, 1 μM Batimastat, or aprotinin (50 μg per lattice). Lattice diameter (mm) was measured daily after seeding (A). The degradation of collagen lattices was determined by the measurement of hydroxyproline released into the culture medium and expressed as nmol per lattice (B). Hydroxyproline was measured using a fluorometric method [51].

with $K_i = 6.03 \pm 0.77$ μM. Taking into account those pleiotropic effects of unsaturated fatty acids on the plasmin and MMP systems, we thus compared their influence on the retraction and degradation of human gingival fibroblast populated collagen lattices with that of aprotinin and Batimastat, known inhibitors of plasmin and MMP activity, respectively [4,10,11,15]. EA (50 μM), but not SA (50 μM), inhibited both lattice retraction and collagen degradation in a time-dependent manner (Fig. 9A and B). Following 6 days of culture, EA reduced lattice retraction and collagenolysis by 28 and 42%, respectively. In comparison, Batimastat reduced lattice retraction and collagenolysis by 40 and 56%, respectively and aprotinin by 68 and 64%, respectively.

4. Discussion

Many active site-directed MMP inhibitors have been tested as potential therapeutic agents in cancer, cardiovascular diseases and osteoarthritis [4,6,52]. The notion of protease exosite inhibitors was recently introduced, i.e. targeting a site remote from enzyme active site but which is essential for substrate recognition [53]. Within the matrixin family, both the hemopexin domain (e.g. in MMP-1 and MT1-MMP) and FnII domains (e.g. in gelatinases), have been suggested as suitable targets for the design of exosite inhibitors [53,54]. Blocking of these sites may provide

more specific inhibition of protease activity than has hitherto been achieved with active site-directed inhibitors. We previously reported that the FnII domains, particularly FnII-1, are involved in the inhibition of gelatinase A by long chain unsaturated fatty acids [27]. Similarly to FnII, the kringle domains of blood coagulation and fibrinolytic proteinases mediate the interaction of these enzymes with other proteins [30–36]. Although having only low sequence homology, both types of module exhibit similar NMR spectroscopic characteristics [31,38]. In keeping with earlier data from Higazi *et al.* [28], we hypothesised that fatty acid binding to kringle domains might interfere with prostromelysin-1 activation. MMP-3 possesses a broad spectrum of activity and can activate several other proMMPs [9,12,14].

Here we show that long chain unsaturated fatty acids, but not their saturated counterparts, significantly interfere with plasmin-mediated proMMP-3 activation. This effect was found to depend upon the number of carbons ($c > 16$) but was influenced neither by the *cis*/*trans* fatty acid configuration nor by the number or positions of double bonds. Similar structure–function relationships were observed in other biological effects exerted by fatty acids but the molecular mechanisms involved in those interactions remain mostly ignored [27,55]. Since inhibition of proMMP-3 activation could be nearly totally abolished by lysine analogues as 6-AHA, or more potently by AMCHA, known to interact with kringle domains [37,56], we postulated that these enzyme exosites are involved in the inhibition of plasmin-mediated proMMP-3 activation. Kringle domains have been previously suggested to play a role in inhibition of plasmin-mediated fibrinolysis by fatty acids [28]. Thus, fatty acids are able to bind to both FnII and kringle domains of gelatinase A and plasmin, respectively, inhibiting the proteolytic activity of those enzymes. β loops in both domains contain a shallow hydrophobic cavity whose size is consistent with accommodating oleic acid. This cavity is lined with a series of aromatic side chain residues [38]. By analogy, leukocyte elastase, which is similarly inhibited by long chain unsaturated fatty acids, was previously shown to interact to both the double bound and the carboxylic group of the fatty acid via Phe^{192} located within the enzyme's extended hydrophobic active site and Arg^{217} at its periphery [57].

Although exosite repeats, such as FnII and kringle domain, share structural analogy, they should not be considered as equivalent in terms of substrate binding properties and cooperativity between domains has been described. For example, $FnII_{1,2}$ are unable to interact with denatured type II, IV and V collagens whereas $FN_{2,3}$ does [58,59]; also $FnII_1$ plays an essential role in elastin and unsaturated fatty acid binding [58,59]. The data presented here suggest a preferential but probably not exclusive interaction between long chain unsaturated fatty acids and the K5 domains in plasmin. (i) SPR analysis demonstrated that plasmin as well as K5 bound OA with a 10-fold higher affinity than LBS-1/OA binding. We noted that the affinity of OA to plasminogen vs. plasmin was better. We can only hypothesized that the conformation or the amino terminal peptide of plasminogen could favour the binding of OA. (ii). This interaction was preferentially inhibited by AMCHA, which has a 4-fold higher affinity for K5 than AHA [36,54], and also by benzamidine which was reported to interact essentially with K5 [60,61]. (iii) The rate of S-2251 hydrolysis by plasmin and miniplasmin but not microplasmin, devoid of K5 was enhanced by OA. (iv) Finally, inhibition by OA of proMMP-3 activation by miniplasmin but not microplasmin confirms the importance of K5 as the main plasmin target in OA binding. However, we can not exclude a possible interaction between fatty acids and kringle 4, since co-operativity of OA binding to LBS or between LBS as been already reported [29,62]. Chemically modified dextrans, carrying a benzylamide group, were also recently shown to modulate plasmin activity through binding to K5 of plasminogen [63]. Although inhibition of gelatinase A activity and plasmin-mediated proMMP-3 activation were obtained at similar OA concentrations, SPR analysis revealed this fatty acid exhibited a nearly 1000-fold higher affinity for K5 as compared to FnII-1. This suggests that as with the first FnII module of gelatinase A, OA nucleation at K5 is an important factor in the inhibition of proMMP-3 activation. Also, since only partial inhibition of activation could be achieved *in vitro* at any OA concentration, other sites besides K5 might be involved in plasmin-mediated proMMP-3 activation, as already suggested by Arza *et al.* [39].

Plasminogen is known to adopt several conformational states: an inactive closed (α) conformation resulting from interaction of the N-terminal peptide of the zymogen mainly with K5, and more extended conformations [62,64–66]. Those latter conformations can be stabilized by binding of lysine analogues that consequently, enhance plasminogen activation and plasmin amidolytic activity. It was already reported [29], that OA enhanced the amidolytic activity of plasmin on its chromogenic substrate S-2251 and this effect was associated with binding of OA to one or more of the LBS in plasmin. On the contrary, the proteolytic activity of plasmin on fibrin that is dependent upon the binding of fibrin to LBS is inhibited by OA [28]. We confirm here that OA enhances the amidolytic activity of plasmin on S-2251 but as for fibrin, the proteolytic activity of the enzyme on proMMP-3 is inhibited. Those apparent opposite effects are explained by the fact that OA competes with LBS for the binding of protein substrate on plasmin that impaired further proteolytic process whereas small chromogenic substrate like S-2251 which does not interact with LBS can be still hydrolysed. The increase of amidolytic activity by OA can be explained by conformational changes in plasmin following the binding of OA to LBS that can favour the cleavage of S-2251 by plasmin. Indeed, interaction of ligands such as fibrin and small

lysine analogues with LBS have been demonstrated to induce conformational changes in plasminogen and kringles 1–5 [64] which further facilitates plasminogen activation to plasmin [67]. In addition, as previously reported [28], low-molecular mass substrates protect plasmin against autodigestion in the presence of OA that results in a stimulation of plasmin chromogenic activity by OA [29]. We also confirm here, using a solid-phase assay system, that the processing of plasminogen by u-PA was increased more than 40-fold following interaction of *LBS* with unsaturated fatty acids. This result suggests that such fatty acids could induce conformational change in native plasminogen that render it more susceptible to u-PA activation as previously reported for other compounds such as 6-AHA [66]. Such conformational changes in plasminogen following binding of OA to LBS can also explain the generation of an amidolytic activity on S-2251 without further processing of plasminogen that consequently can favour the activation of plasminogen to plasmin by u-PA as it was observed in our study. Overall, these data indicate that plasmin-mediated proMMP-3 activation and subsequent proteolytic cascades may depend on the relative concentrations of plasminogen activators, plasminogen and unsaturated fatty acid. Two different cell types were used to evaluate the inhibitory influence of unsaturated fatty acids on the MMP-3/MMP-1 proteolytic cascade triggered by plasmin: dermal fibroblasts that produce very low levels of plasminogen activators under basal conditions, and gingival fibroblasts that express both t-PA and u-PA in keeping with the increased healing capacity of gingiva as compared to skin [50,51]. Supplementation of cell culture medium with exogenous plasmin was therefore necessary to induce MMP-3/MMP-1 activation cascade in dermal fibroblasts while addition of plasminogen could directly trigger this cascade in their gingival counterparts. Elaidic acid was found to be as effective as *in vitro* in inhibiting proMMPs processing in both of these models. Besides acting as regulators of plasmin-mediated proMMP-3 activation, long chain unsaturated fatty acids can inhibit MMP-3 activity. Although those fatty acids exhibit preferential binding mode to gelatinase A FnII domains, they may also interact with the MMP active site since they are able to inhibit MMP-2 deleted of its FnII exosites [27]. We verified this observation using a truncated form of MMP-3, devoid of both pro- and hemopexin domains, and found that EA inhibited MMP-3 activity with K_i values in the micromolar range. This higher MMP-3 inhibitory capacity as compared to MMP-1, might be related to the deep hydrophobic and flexible characteristics of stromelysin-1 S1′ cavity [52]. Interestingly, polyunsaturated fatty acids, as *cis*- or *trans*-parinaric acid, also displayed potent MMP-3 inhibitory capacity and in preliminary investigations n-3 fatty acids were also found to suppress MMP activity.

To directly assess the consequence of the multiple effects of unsaturated fatty acids on MMP activation and activity, we used gingival fibroblast-populated collagen lattice as a model system. Both contraction of lattices and collagen degradation were inhibited by EA at a level similar to that reached by potent plasmin or MMP inhibitors i.e. aprotinin and Batimastat. This suggested that naturally occurring fatty acids could advantageously compensate for any TIMP deficiency. In the context of exposure of human skin to UV light, where MMP activation and activity are not fully controlled by TIMPs [68], unsaturated fatty acids or molecules with similar structures could be attractive target for the development of pharmacological inhibitors. Our data may also shed light on vascular diseases such as atherosclerosis where increased expression and activation of MMP-3 and MMP-1 is correlated with an increased risk of plaque rupture [69,70]. Unsaturated fatty acids such as OA are found in the circulation [71] and it is conceivable that specific diets like the Mediterranean diet, where OA represents the main source of lipids, could exert their reported protective effects against vascular diseases and cancer [72–74] by OA inhibition of MMP activation and activity.

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