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Thrombin induces rapid PAR1-mediated non-classical FGF1 release

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Abstract

Thrombin induces cell proliferation and migration during vascular injury. We report that thrombin rapidly stimulated expression and release of the pro-angiogenic polypeptide fibroblast growth factor 1 (FGF1). Thrombin failed to induce FGF1 release from protease activated receptor 1 (PAR1) null fibroblasts, indicating that this effect was dependent on PAR1. Similarly to thrombin, FGF1 expression and release were induced by TRAP, a specific oligopeptide agonist of PAR1. These results identify a novel aspect of the crosstalk between FGF and thrombin signaling pathways which both play important roles in tissue repair and angiogenesis.

Keywords

thrombin; PAR1; FGF1; non-classical release

Abbreviations

bovine calf serum, BCS; cell lysate, CL; Dulbecco's modified Eagle's medium, DMEM; fibroblast growth factor 1, FGF1; Mouse brain endothelial cells, MBEC; mouse embryo fibroblasts, MEF; lactate dehydrogenase, LDH; protease activated receptor 1, PAR1; thrombin receptor-activator peptide, TRAP

1. Introduction

Serine protease thrombin, a key enzyme of the blood coagulation cascade, stimulates cell proliferation and motility and regulates tissue repair and angiogenesis [1,2]. Thrombin mediates its cell responses through the activation of protease-activated receptors (PAR) [1,2]. The expression of several growth factors [3–6] is induced in response to thrombin, including fibroblast growth factor (FGF) 2 [3,4], platelet-derived growth factor [5], and vascular endothelium growth factor [6].

Similarly to thrombin, FGF1 is involved in angiogenesis and tissue repair [7,8]. FGF1 lacks a classical signal peptide sequence and is exported through a stress-dependent non-classical ER-Golgi-independent pathway [9]. We found that thrombin stimulated the expression and release of FGF1 under non-stress conditions. The analysis of FGF1 release from thrombin-treated PAR1 null cells demonstrated that FGF1 export was PAR1-dependent. Our findings revealed a novel interplay between thrombin signaling and non-classical FGF1 release, which may be important for vascular remodeling.

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2. Materials and Methods

Cell culture

NIH 3T3 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% Bovine Calf Serum (BCS; Hyclone) and a 1x antibiotic-antimycotic mixture (Life Technologies, Inc.). PAR1 null mouse embryonic fibroblasts (MEF) and PAR1 null MEF transfected with PAR1 were grown in DMEM supplemented with 10% Fetal Bovine Serum (Hyclone). The cells used in the FGF1 release experiments were grown on human fibronectin-coated (10 $\mu\text{g}/\text{cm}^2$) dishes [10].

DNA constructs, stable FGF1R136K NIH 3T3 transfectants, preparation of adenovirus, adenoviral transduction, RT-PCR

A thrombin-resistant FGF1 mutant was constructed by site-directed mutagenesis of the FGF1pMEXneo plasmid [10]. The codon encoding arginine 136 (AGA) was changed to a lysine (AAA). NIH 3T3 cells were transfected with FGF1R136K using the FuGene (Roche) reagent, and further selected by using geneticin (GIBCO). FGF1R136K was additionally cloned in the pAdlox shuttle vector. The corresponding adenovirus was prepared; and PAR1 null, as well as PAR1-transfected MEF, were transduced by incubation in serum-free medium with the FGF1R136K or the wild type FGF1 adenovirus, as described [11]. The expression of PAR1 in MEF was assessed by RT-PCR using specific primers (5'-GAACAAAGCCCCGCGACTTC-3' and 5'-CTGATTGGCAGTTCGGGTC-3').

Analysis of FGF1 release and expression

Stable FGF1R136K NIH 3T3 cell transfectants or adenovirally transduced MEF were washed with serum-free DMEM containing 5 U/ml of heparin (Sigma), and stimulated with either 1 U/ml of thrombin or 5.7 μM thrombin receptor-activator peptide (TRAP, Sigma) at 37°C for different time periods. In some experiments, a KTLLQN hexapeptide (New England Peptides) at 5.7 μM concentration was used as a negative control for TRAP. Alternatively, the release of FGF1 was stimulated by the incubation of cells at 42°C for 110 minutes as previously described [10]. Conditioned media and cell lysates were collected and tested for FGF1 content by heparin chromatography and immunoblot analysis [10]. The processed conditioned medium from one 150 mm cell culture dish was used at each time point. Unless otherwise indicated, 1/10 of the cell lysate derived from one 150 mm plate was loaded to each gel for control of FGF1 expression. Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity in conditioned medium after filtration [12]. Nuclear run-on analysis of FGF1 expression in NIH 3T3 cells was performed as previously described [13]. Plasmid constructs (0.4 $\mu\text{g}/\text{dot}$) containing either the *fgf1* or *gapdh* cDNA were used as probes. Densitometric analysis of the radioautographs and Western blots was performed utilizing a phosphorimager (Molecular Dynamics).

Immunofluorescence confocal microscopy and preparation of cell membranes

NIH 3T3 cells growing on coverslips were transfected with FGF1:HA cloned in the pCR3.1 vector, using the FuGene reagent. The following day, the cells were stimulated for 5 or 30 minutes with 1 U/ml of thrombin, then fixed with formaldehyde, incubated with the monoclonal anti-HA antibodies (Covance) in permeabilizing (0.1% Triton X100 and 0.1% Tween 20) or non-permeabilizing buffer, followed by FITC-conjugated anti-mouse IgG antibodies (Vector Laboratories) and Hoechst 34580 (Molecular Probes), and studied using the LTCS-SP confocal microscope (Leica).

To isolate the cell membranes, FGF1R136K NIH 3T3 cells were stimulated with 1 U/ml of thrombin at 37°C for 30 minutes. The cells were washed in PBS, scraped, and quickly spun

down. The cell pellets were resuspended in 1 ml of hypotonic solution (2.5 mM HEPES pH 7.0, 25 mM sucrose, and protease inhibitor cocktail (Roche)), and incubated on ice for 20 minutes. The pellets were homogenized in a Dounce homogenizer and centrifuged for 10 minutes at 10,000 g, 4°C. The supernatants were then collected and centrifuged at 40,000 rpm for 18 hours, to precipitate the membranes.

3. Results

Because thrombin induces the expression of several growth factors [3,5,6,14], we explored its ability to stimulate the transcription of FGF1. The expression of the FGF1 transcript in NIH 3T3 cells in response to thrombin was analyzed by nuclear run-on assay. The induction of FGF1 mRNA transcription was initially detected 15 minutes after the addition of thrombin to NIH 3T3 cells and increased over time (Figure 1A).

Since thrombin induced FGF1 expression (Figure 1A), we attempted to determine whether it induces FGF1 release. Because the level of endogenous FGF1 in NIH 3T3 cells after thrombin stimulation was below the sensitivity of immunoblotting, we used cells transfected with FGF1, as we previously did in our studies of stress-induced FGF1 export [9]. FGF1 is susceptible to thrombin cleavage at arginine 136 [15]; therefore, we utilized a thrombin-resistant FGF1 mutant (FGF1R136K). The addition of thrombin to FGF1R136K NIH 3T3 cell transfectants at 37°C resulted in the rapid and sustainable appearance of FGF1R136K in the extracellular compartment (Figure 1B). Conversely, similarly to wild type FGF1 [10], the release of the FGF1R136K mutant required 90 minutes of temperature stress at 42°C in order to be detected by immunoblotting (data not shown). Similarly to heat shock, thrombin treatment did not induce the release of LDH from the cells (data not shown); thus, neither treatment resulted in cell damage. The release of the FGF1R136K mutant was dependent on the concentration of thrombin and the maximal response was achieved at 1 U/ml (10 nM) (Supplementary Figure 1). This concentration is physiologically relevant, because the level of prothrombin in circulation is 1–2 μ M and fibrin clot formation occurs at 10–30 nM thrombin concentration [16]. The rapid thrombin-induced release of FGF1 did not require protein synthesis. Indeed, cycloheximide (10 μ g/ml) treatment did not interfere with FGF1 release stimulated for 5 minutes with thrombin (Figure 1C).

Heat shock induces the translocation of FGF1 to the cell periphery [17]. To evaluate whether thrombin induces the association of FGF1 with the cell membrane, we performed subcellular fractionation of FGF1R136K NIH 3T3 cell transfectants treated or untreated with thrombin for 30 minutes at 37°C. Thrombin induced the appearance of FGF1R136K in the membrane fraction (Figure 2A). In the next series of experiments, using immunofluorescence confocal microscopy, we observed that 30 minutes thrombin treatment of NIH 3T3 cells transiently transfected with FGF1:HA resulted in the translocation of FGF1 to the cell periphery (Figure 2B, iii). Interestingly, immunofluorescence confocal study of cells treated with thrombin for 5 minutes did not reveal the peripheral redistribution of FGF1 (Figure 2 B, ii), although this duration of treatment was sufficient to induce FGF1 export (Figure 1B). To specifically detect released FGF1 associated with the outer surface of the cell membrane, we omitted permeabilizing detergents from the immunofluorescence staining buffer. As a result, we were able to see that 5 minutes of treatment was sufficient to induce the appearance of FGF1:HA on the cell surface (Figure 2B, v). Apparently, thrombin enabled the immediate export of FGF1 from the FGF1 fraction localized in the vicinity of the cell membrane. Continued FGF1 export may be provided by a thrombin-dependent peripheral redistribution of FGF1, which is a slower process.

Since thrombin mediates its biological responses predominantly through the activation of PAR [18], we evaluated the role of PAR1 in thrombin-induced expression and release of FGF1. We

utilized PAR1 null mice-derived MEF [19] that were transduced with the FGF1R136K adenovirus. The absence of PAR1 expression in PAR1 null MEF was confirmed by RT-PCR (Supplementary Figure 2). Thrombin was unable to induce export of the FGF1R136K from PAR1 null MEF (Figure 3A, upper panel). However, PAR1 null cells exported FGF1R136K in response to temperature stress, suggesting that they were not defective in mediating stress-induced non-classical FGF1 export (Figure 3A, upper panel). Conversely, thrombin induced rapid FGF1R136K release from control PAR1 null MEF transfected with PAR1 (Figure 3A, lower panel). PAR1-activating peptide TRAP, similarly to thrombin, induced the rapid expression of endogenous FGF1 in NIH 3T3 cells (Figure 3B). It also induced the export of FGF1R136K from NIH 3T3 cells (Figure 3C), and FGF1 from PAR1-transfected MEF (Supplementary Figure 3, left panel), while PAR1 null MEF did not export FGF1 in response to TRAP (Supplementary Figure 3, right panel). Moreover, a control hexapeptide KTLLQN did not stimulate FGF1 export from PAR1-transfected MEF (Supplementary Figure 3, left panel). Thus, rapid stimulation of FGF1 expression and release by thrombin were dependent on PAR signaling.

4. Discussion

FGF1 participates in vascular remodeling and promotes angiogenesis after vascular injury [8]. Tissue injury is often accompanied by a thrombotic event. Thrombin at sites of injury may play a critical role, not only in the formation of the fibrin matrix but also in the stimulation of expression and export of FGF1 as well as expression of other [3–6] growth factors. The export of FGF1 was detected as early as 5 minutes after thrombin stimulation, and this effect did not require protein synthesis. Apparently, the release of FGF1 can be a rapid reaction to tissue damage, and it could contribute to tissue repair. FGF1 contains a thrombin cleavage site [15]; however, since this cleavage is inhibited by heparin [20], we suggest that extracellular heparan sulfates can protect the released FGF1 from thrombin-induced cleavage, thus insuring FGF1 activity at the sites of vascular injury. Interestingly, thrombin has recently been reported to induce the increase of the extracellular pool of another non-classically secreted growth factor [21–23], FGF2 [24]. Based on the rapid dynamics of this effect (15 minutes after thrombin stimulation) it does not appear to be a result of the induction of FGF2 expression by thrombin, and it is most probably due to enhanced FGF2 release.

PAR1 promotes inflammation and abnormal remodeling during restenosis, neointima formation after vascular injury, and fibrosis in the injured lung [2]. We found that rapid stimulation of FGF1 expression and release by thrombin was dependent on PAR1. We suggest that PAR1-dependent abnormal tissue remodeling is at least partially mediated by FGF1 release.

The presented results raise a number of questions to explore in the future: 1. What is the role of FGF1 expression and release in thrombin- and PAR1-induced cell proliferation *in vivo*? 2. Which signaling pathways downstream of PAR1 are responsible for FGF1 expression and release? 3. Do the components of stress-induced FGF1 release, such as the small calcium-binding protein S100A13, synaptotagmin 1, and copper ions [9] participate in the thrombin-induced FGF1 export?

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Thrombin induces FGF1 expression. The expression of *fgf1* in thrombin-treated NIH 3T3 cells was evaluated by nuclear run-on analysis [13]. The bar graphs represent the normalized ratio of [32 P]-labeled *fgf1* to the *gapdh* transcript $\pm$ s.e.m. Mouse brain endothelial cells (MBEC) that express endogenous FGF1 served as a positive control. **B. Thrombin induces rapid release of FGF1R136K at 37°C.** FGF1 immunoblot analysis was used to identify FGF1R136K in media conditioned by FGF1R136K NIH 3T3 cell transfectants. Media conditioned in the absence of thrombin at 37°C or by heat shocked cells (42°C, 110 minutes) served as controls. Cell lysate (CL) from these cells is shown in the left panel (1/10 of total cell lysate was loaded). Bar graphs represent the percentage of FGF1 released from FGF1R136K NIH 3T3 cell transfectants upon thrombin treatment. The densitometric values for different time points were normalized for total FGF1 expression levels. Each bar represents the mean of the normalized FGF1 release $\pm$ s.e.m. from three independent experiments. **C. Thrombin-induced FGF1 release is not sensitive to the inhibition of protein synthesis.** FGF1 immunoblot analysis of media conditioned by FGF1R136K NIH 3T3 transfectants in response to 5 minutes of 1 U/ml thrombin treatment, in the presence or absence of 10 μ g/ml cycloheximide (Sigma) added to the cells 15 minutes before thrombin stimulation.

Figure 2.

A. Thrombin-induced association of FGF1 with the cell membrane. FGF1R136K NIH 3T3 cells transfectants were incubated with or without 1 U/ml of thrombin for 30 minutes; the cytosol and membrane fractions were prepared as described in the “Materials and Methods”. 100% of cytosol and membrane fractions were loaded to the gel. Immunoblotting was used to detect FGF1. **B.** Thrombin-induced peripheral redistribution of FGF1 and appearance of FGF1 on the cell surface. FGF1:HA transfectant NIH 3T3 cells, unstimulated (i, iv) or stimulated with 1 U/ml of thrombin for 5 minutes (ii, v) or 30 minutes (iii, vi), were fixed and processed for immunofluorescence microscopy as described in the “Material and Methods” in permeabilizing (i, ii, iii) or non-permeabilizing (iv, v, vi) conditions. Confocal images of median horizontal cell sections were taken using the 100X objective. Green - FGF1:HA, blue - DNA. Bar 20 μ m.

Figure 3.

A. PAR1 mediates rapid thrombin-induced release of FGF1. FGF1 immunoblot analysis of media conditioned by PAR1 null or PAR1-transfected PAR1 null MEF adenovirally transduced with FGF1R136K and stimulated with thrombin was performed as described in Figure 1B.

B. TRAP rapidly induces FGF1 expression. The expression of *fgf1* in NIH 3T3 cells treated with 5.7 μM TRAP was evaluated by nuclear run-on analysis, as described in Figure 1A.

C. TRAP rapidly induces FGF1 release. FGF1 immunoblot analysis of media conditioned by FGF1R136K NIH 3T3 cell transfectants, in response to the addition of TRAP, was performed as described in Figure 1B. 1/20 of total cell lysate was loaded to the gel in these experiments.