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Shear Stress Induces the Release of an Endothelial Elastase: Role in Integrin $\alpha_v\beta_3$-Mediated FGF-2 Release

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Key Words
Shear stress · Mechanotransduction · Elastase · Integrin $\alpha_v\beta_3$ · Fibroblast growth factor 2

Abstract
Background/Aims: Laminar shear stress is an important stimulus in the endothelium-dependent control of vascular tone and of vascular remodeling processes. Based on previous studies demonstrating integrin-mediated release of fibroblast growth factor 2 (FGF-2), we investigated whether shear stress-induced integrin activation requires the involvement of an extracellular protease. ***Methods:*** Cultured porcine aortic endothelial cells (PAEC) were exposed to laminar shear stress (16 dyn/cm^2), whereas static cells served as controls. ***Results:*** Exposure of PAEC to shear stress led to an increased activity of a protease in supernatants. This protease could be characterized as elastase but was different from neutrophil and pancreatic elastases. The enhanced activity was accompanied by the activation of integrin $\alpha_v\beta_3$ and p38 MAPK, and followed by an increased FGF-2 concentration in the supernatant. Pretreatment with inhibitors of either elastase or integrin $\alpha_v\beta_3$ resulted in a reduction of FGF-2 release. The observed effects of shear stress on integrin $\alpha_v\beta_3$ and p38 MAPK activation, as well as on FGF-2 release could be mimicked by application of pancreatic elastase to static endothelial cells. ***Conclusion:*** By inducing the release of an endothelial elastase, shear stress induces an integrin-dependent release of FGF-2 from endothelial cells.

Introduction

Fluid shear stress is probably the most significant physiological stimulus for endothelial cells to produce the endothelium-derived autacoids nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor [1, 2]. Functionally, this shear stress-induced release of autacoids elicits a flow-dependent dilation allowing the acute adaptation of vascular conductivity to an augmented flow load. Moreover, shear stress controls the expression of various genes involved in autacoid synthesis and, in addition, cell survival, cell growth and cell cycle control [3–5]. Consequently, shear stress is also a pivotal stimulus for structural arterial remodeling following chronic alteration of blood flow [6–8].

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There is ample evidence that shear stress stimulates endothelial cells via multiple pathways, involving the activation of ion channels, G protein-coupled receptors, integrins as well as receptor tyrosine kinases, ultimately leading to the activation of mitogen-activated protein kinases (MAPK) and downstream transcription factors. Integrins, membrane caveolae, receptor tyrosine kinases, G proteins, ion channels as well as glycocalyx components have been reported to act as mechanotransducers upon endothelial stimulation by shear stress [9–11]. Considering that a mechanical deformation of the cells occurs during exposure to shear forces, the cytoskeleton and its plasmalemmal anchoring complexes also play a role in the mechanotransduction of shear stress [12]. In particular, transmembrane proteins such as platelet endothelial cell adhesion molecule (PECAM-1) and integrins potentially act as mechanotransducers. With regard to integrins, there is evidence for inside-out signaling during shear stress which may support endothelial cell adherence under conditions of elevated external forces. Indeed, PECAM-1, using vascular endothelial cell cadherin as an adaptor molecule, elicits activation of integrins in a classical inside-out manner [13]. However, there is also evidence that shear stress leads to outside-in integrin signaling. Several reports on altered expression, activity and release of metalloproteinases and cysteine proteases (cathepsins L and K) under shear stress strongly suggest that modulation of the extracellular matrix (ECM) by these enzymes contributes to activation of cellular adhesion molecules as part of mechanotransduction [14–17]. Moreover, a modulation of ECM protein expression under chronic shear stress has been reported [18, 19]. Besides, various different ECM proteins or their fragments can lead to specific integrin and MAP kinase signaling during flow [20]. Therefore, the production, composition and in particular the enzymatic modification of the ECM may be pivotal in the response of endothelial cells to shear stress.

We have shown previously that endothelial adhesion molecules, in particular laminin-binding protein and integrin $\alpha_v\beta_3$, mediate shear stress responses in cultured endothelial cells [21, 22]. Laminin-binding protein has been shown to act as an essential mediator of shear stress-induced endothelial nitric oxide synthase expression in porcine aortic endothelial cells. In addition, integrin $\alpha_v\beta_3$ was found to be an integral part of the signaling cascade leading to shear stress-induced release of fibroblast growth factor 2 (FGF-2) from endothelial cells, a potentially significant process in flow-related vascular remodeling. However, the mechanism of integrin activation remained unclear.

Based on previous observations by others on the occurrence of proteases or their inhibitors during shear stress [14–17], we hypothesized that the activation of the integrin $\alpha_v\beta_3$, and hence the FGF-2 release, is due to the action of an endothelium-derived protease. Therefore, we studied potential alterations of matrix composition and of proteolytic activities in endothelial cell cultures exposed to arterial levels of shear stress. Since we found evidence for the release of a yet unidentified elastase during shear stress, we also studied whether exogenously applied elastase could mimic the effects of shear stress on integrin $\alpha_v\beta_3$ activation and FGF-2 release in static endothelial cells.

Methods

Unless stated otherwise, all reagents were purchased from Applichem.

Cell Culture

Porcine aortic endothelial cells (PAEC) were isolated as described previously by Gloe et al. [21]. Cells were cultured on standard plastic culture dishes in DMEM (Gibco) containing 10% FCS (Biochrom) and 1% of a mixture of amphotericin B, penicillin and streptomycin (Sigma). They were subcultured using trypsin/EDTA (Sigma). Cultured cells were identified as endothelial cells by the presence of PECAM-1 antigen. PAEC at passages 3–5 were used in all experiments.

Shear Stress Experiments

A laminar shear stress of 16 dyn/cm^2 was applied using a cone and plate apparatus as described before [21]. Cells were transferred onto glass plates coated with collagen G (10 μg/ml; Biochrom). After 16 h, the medium was changed to DMEM (without phenol red; Sigma) containing 1% FCS. The inhibitors chymostatin (CHY; Sigma), methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MEO; Bachem) and Abciximab (ABC; Lilly) were added 15 min prior to the exposure to shear stress and left in the medium throughout the experiment. Static controls were taken from the same culture and also grown on glass plates. Afterwards, the supernatant was collected for quantitative FGF-2 and elastase activity measurements. The cells were immediately lysed for Western blot analysis or fixed for immunofluorescence experiments as described below. Protein extracts were frozen after preparation and stored at –20°C until further analysis. Cell lysates were analyzed for equal protein levels using the bicinchoninic acid assay (Perbio).

Assessment of FGF-2

FGF-2 in cell supernatants was measured using a commercially available ELISA kit (R&D Systems) according to the manufacturer's instructions. Immunostaining of FGF-2 was performed as follows: PAEC were fixed with 3.7% formaldehyde, blocked using 1% bovine serum albumin in PBS, and incubated with the primary FGF-2 antibody (Sigma) overnight at 4°C. The FITC-la-

beled secondary antibody (Invitrogen) was applied for 30 min at room temperature. The samples were analyzed using a standard fluorescence microscope (Axiovert 200M; Zeiss). FGF-2 translocation to the cell surface was analyzed by flow cytometry (FACScan; Becton Dickinson) using a FGF-2 primary antibody (Sigma) and an FITC-labeled secondary antibody (Invitrogen). Suspended cells were detached using a citrate buffer composed of 135 mM KCl and 15 mM sodium citrate.

Determination of Elastase Activity

Elastase activity measurements were performed using the specific substrate methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroaniline (MeOSuc-Ala-Ala-Pro-Val-pNA, 20 μg/ml; Bachem). Fifty microliters of each sample was incubated with 1 μl of substrate for 30 min and absorbance detected using a spectrophotometer. A calibration curve for each individual set of experiments was generated using porcine pancreatic elastase.

Zymography

Supernatants from cells exposed to shear stress and static controls were analyzed by SDS-PAGE (gels supplemented with 10 mg/ml gelatine) using denaturing but nonreducing conditions. SDS was removed from the gel by 3 subsequent washings with 2.5% Triton X-100 for 30 min each. Proteases were allowed to react with the gelatine in the gel for 16 h at 37°C in a bathing solution containing 50 mM Tris (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Tween 20. Finally, the gel was stained with Coomassie blue G250 (0.7 mM in 10% acetic acid) and destained (7% acetic acid in 5% methanol) until areas of proteolytic activity were detectable as white bands.

Immunoblotting and Immunoprecipitation

The buffer used for cell lysis was composed as follows: 20 mM Tris, 137 mM NaCl, 2 mM EDTA (pH 8.0), 10% glycerol and 0.1% sodium deoxylcholate. The protease and phosphatase inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml), phenylmethylsulfonyl fluoride (1 mM), sodium fluoride (0.5 mM) and sodium orthovanadate (0.5 mM) were added to the buffer prior to each experiment. Protein content was assessed using a bicinchoninic acid assay (Perbio). Equal amounts of total protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane using standard techniques. The membrane was incubated overnight at 4°C with antibodies against neutrophil elastase (Santa Cruz), Shc (Upstate), phospho-p38 (Cell Signaling), phospho-ERK1/ERK2 (Cell Signaling), GAPDH (Chemicon), p38 (Cell Signaling) and phospho-Hsp27 (Cell Signaling). Incubation with the secondary antibody (Calbiochem) was performed subsequently for 1.5 h at room temperature. Protein bands were detected using a horseradish peroxide chemiluminescence detection kit.

For immunoprecipitation cell lysates were incubated for 2 h at 4°C with LM609 (Chemicon), an antibody to integrin $\alpha_v\beta_3$, and magnetic beads coated with protein G (Miltenyi Biotec). Precipitates were separated using a magnetic separation unit (Miltenyi Biotec). Immunoblotting was performed as described above.

Elastase Experiments

Static cells were treated with porcine pancreatic elastase (0.5 U/ml; SERVA). The elastase was applied to the supernatant of static endothelial cells for 5, 10, 30 or 60 min. Cells were then immediately fixed or lysed as described above. MAP kinase inhibition was performed using the p38 MAPK inhibitor SB201290 (10 μM; Upstate) or U0126 (10 μM; Calbiochem) to block the activity of MEK1/2. These substances were added to the medium 30 min prior to elastase treatment.

Statistical Analysis

All quantitative data are expressed as means ± standard error of the mean. Statistical tests were performed using the SigmaStat statistic software (SPSS Inc.). If not stated otherwise, differences between treatment groups or changes over time were analyzed using one-way analysis of variance for repeated measures (ANOVA RM) followed by post hoc analysis using the Student Newman-Keuls method. If data were not normally distributed, ANOVA RM on ranks was used but mean values are presented nevertheless for comparability of data. Representative immunoblots and fluorescence images are shown. The number of experiments is indicated in the figure legends. p values less than 0.05 were considered significant.

Results

Effects of Shear Stress

An Elastase Is Released by Endothelial Cells after Exposure to Shear Stress

Zymographic analysis revealed a marked proteolytic activity at 28 kDa in supernatants from cells exposed to shear stress but not in static cells (fig. 1a). Supernatants of static and cells exposed to shear stress were additionally analyzed by immunoblotting using an antibody raised against neutrophil elastase. As in zymograms, a band was observed at 28 kDa only after shear stress exposure (fig. 1b). To further characterize this protease activity we used the specific elastase substrate MeOSuc-Ala-Ala-Pro-Val-pNA. Elastase activity in the supernatant of cells exposed to shear stress was more than 3-fold higher than in the supernatant of static controls (static 0.06 ± 0.01 U/ml, shear 0.20 ± 0.03 U/ml) (fig. 1c). An increase in elastase activity could still be observed after pretreatment with ABC, an antibody fragment that blocks the integrin $\alpha_v\beta_3$ (static + ABC 0.04 ± 0.01 U/ml, shear + ABC 0.15 ± 0.01 U/ml). To assess how quickly this protease is released, time course experiments were performed. Significantly increased elastase activity was already detectable after 5 min of exposure to shear stress. During the following 30 min, the elastase concentration in the supernatant increased further, after which it reached a plateau (fig. 1d). In order to investigate whether neutrophil or pancreatic elastases are expressed in endothelial cells, we performed RT-PCR. However, neither neutrophil nor pancreatic elastases were revealed in endothelial cells at the mRNA level (data not shown).

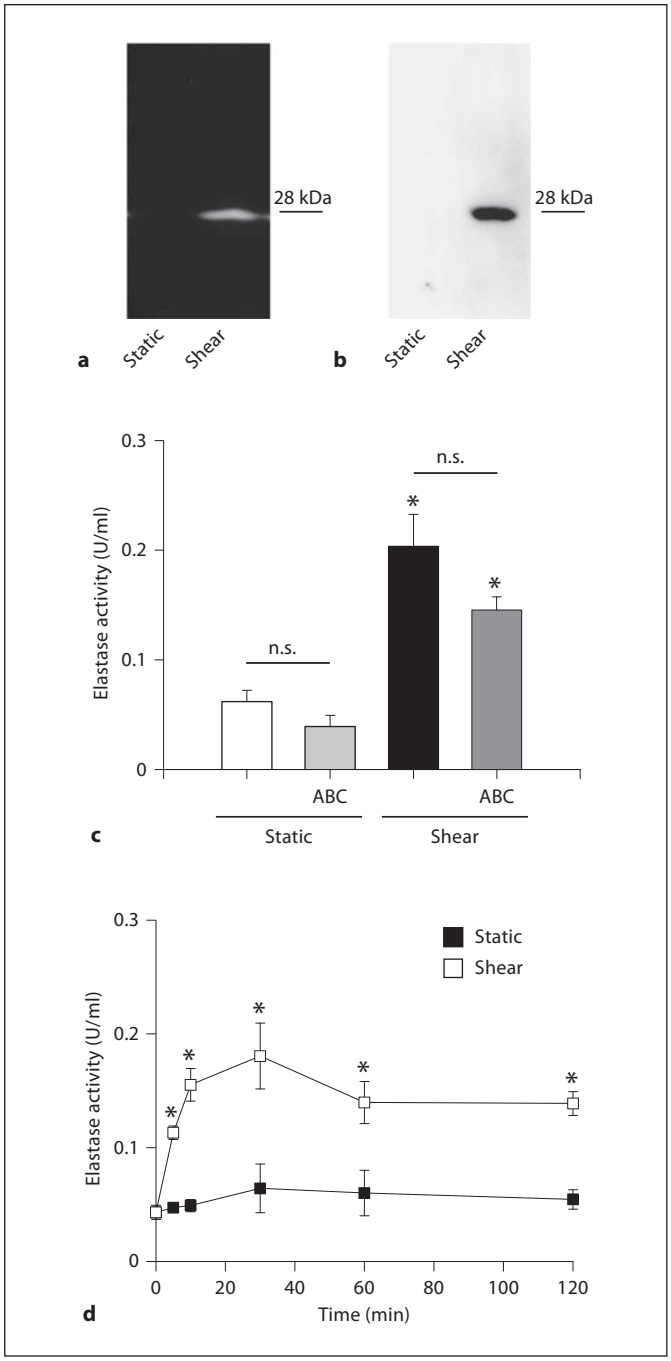

Fig. 1. Endothelial shear stress is associated with an increased elastase activity in the supernatant. **a** Supernatants of static and cells exposed to 16 dyn/cm^2 shear stress (2 h) were analyzed by zymography (n = 3). White bands represent areas of protease activity. **b** Supernatants were analyzed by immunoblotting using an anti-neutrophil elastase antibody (n = 3). **c** Enzymatic activity in supernatants was determined using MeOSuc-Ala-Ala-Pro-Val-pNA (ABC, 1 μg/ml; n = 20). * $p < 0.05$ vs. static, ANOVA RM on ranks, Student-Newman-Keuls. n.s. = Not significant. **d** A significant increase of elastase activity occurred already after 5 min of shear stress exposure (n = 4–6). * $p < 0.05$ vs. static.

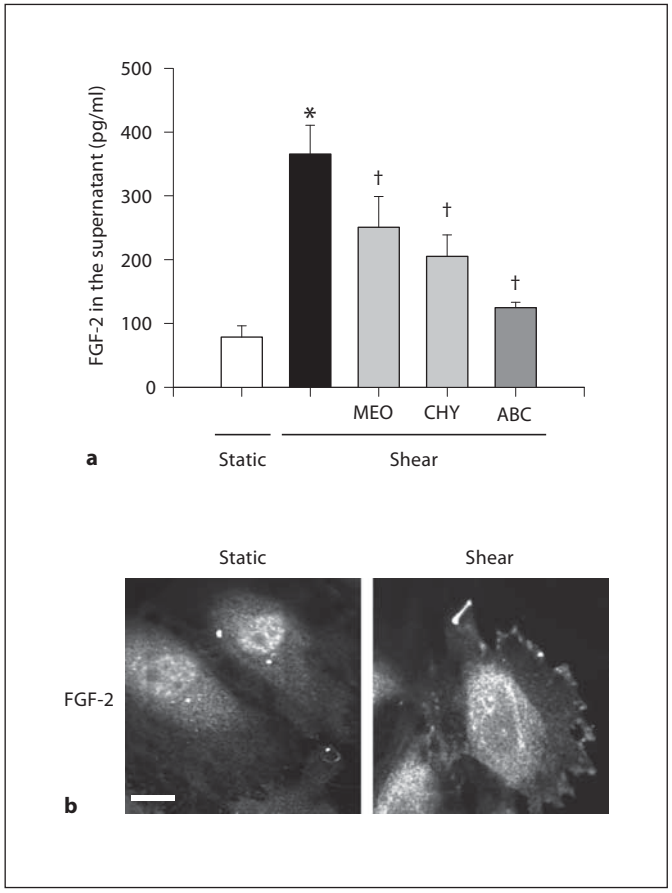

Fig. 2. The effect of shear stress on FGF-2 release is dependent on elastase and the integrin $\alpha_v\beta_3$. **a** PAEC were either left static or exposed to 16 dyn/cm^2 shear stress for 2 h. Serine protease inhibition was performed using CHY (10 μg/ml), specific elastase inhibition with MEO (50 μM) and integrin $\alpha_v\beta_3$ was inhibited with ABC (1 μg/ml) (n = 7–12). * $p < 0.001$ vs. static; † $p < 0.05$ vs. shear. **b** FGF-2 immunofluorescence images of static cells and cells exposed to shear stress show a translocation of FGF-2 to the cell membrane (16 dyn/cm^2, 2 h, n = 3). Scale bar = 10 μm. ×63.

Shear Stress-Induced FGF-2 Release Is Elastase Dependent

Shear stress not only increased elastase activity in the supernatant but also significantly augmented the concentration of FGF-2 in the supernatant more than 4-fold (static 78.5 ± 17.9 pg/ml, shear 365.5 ± 45.1 pg/ml; fig. 2a), which is in line with our previous findings [22]. This increase in FGF-2 concentration was partly an elastase-dependent effect, since pretreatment with both the unspecific serine protease inhibitor CHY (10 μg/ml,

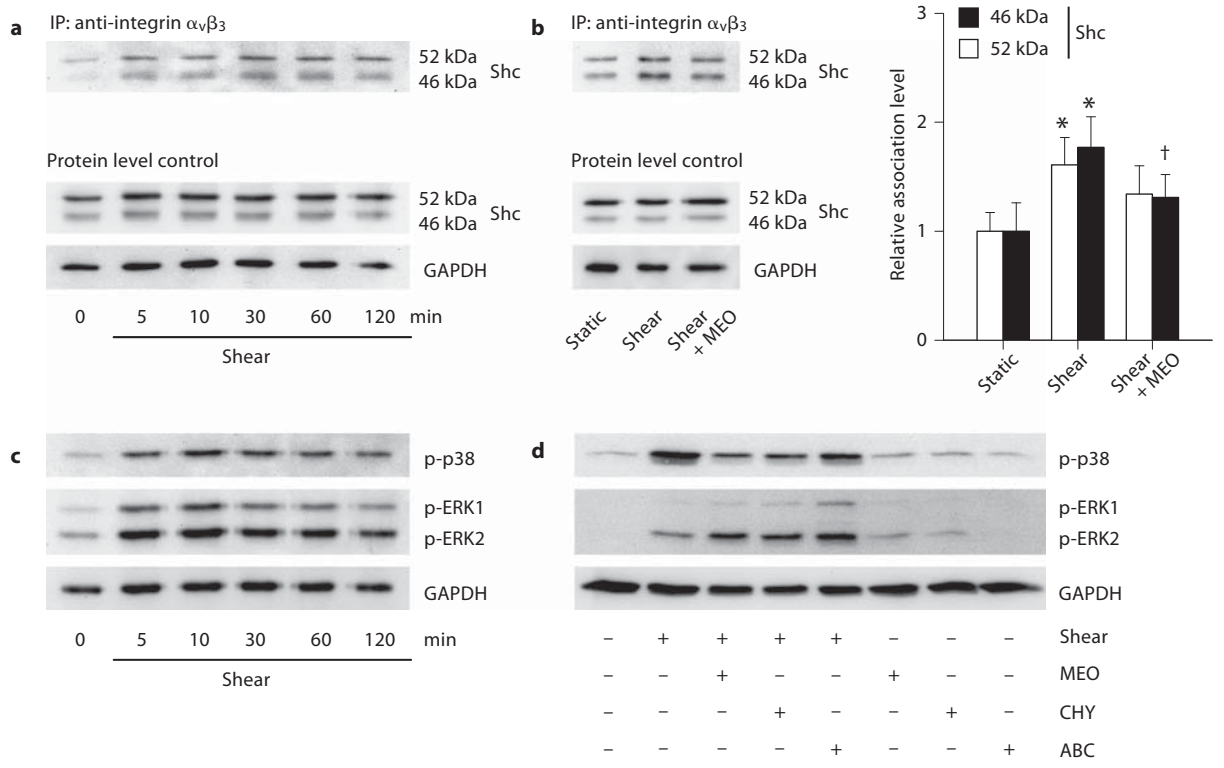

Fig. 3. Elastase inhibition decreases shear stress-induced integrin $\alpha_v\beta_3$ and p38 MAPK activation. **a**, **b** PAEC lysates were subjected to immunoprecipitation (IP) with an $\alpha_v\beta_3$ antibody and immunoblotted against Shc. Cell lysates were separately immunoblotted for Shc and GAPDH to confirm equal protein loading. **a** Shear stress (16 dyn/cm^2) induced a sustained increase in the association of integrin $\alpha_v\beta_3$ with Shc (n = 3). **b** Elastase inhibition (MEO, 50 μM) reduced this interaction (16 dyn/cm^2, 1 h; n = 4). * $p < 0.05$ vs. static; † $p < 0.05$ vs. shear. **c** Static PAEC or cells exposed to 16 dyn/cm^2 shear stress were analyzed for p38 MAPK and ERK activation by immunoblotting (n = 3). **d** Elastase activity was inhibited using MEO (50 μM) or CHY (10 μg/ml). The integrin $\alpha_v\beta_3$ was blocked with ABC (1 μg/ml) (n = 3). GAPDH was used to control equal protein loading.

shear + CHY 205.3 ± 33.7 pg/ml) as well as the specific elastase inhibitor MEO (shear + MEO 250.8 ± 48.2 pg/ml) significantly attenuated the shear stress-induced FGF-2 increase. In accordance with our earlier study [22], blocking the integrin $\alpha_v\beta_3$ with ABC abolished the increase in FGF-2 in supernatants during shear stress (shear + ABC 124.9 ± 8.4 pg/ml; fig. 2a).

Immunofluorescence labeling of static cells showed FGF-2 mainly in nuclear and perinuclear regions. In contrast, shear stress-treated cells additionally exhibited aggregate-like staining of FGF-2 at the plasma membrane, suggesting that part of the intracellular FGF-2 had been redistributed towards the membrane for release upon shear stress exposure (fig. 2b).

Shear Stress-Induced Integrin $\alpha_v\beta_3$ Activation Is Elastase Dependent

To determine whether the activation state of the integrin $\alpha_v\beta_3$ was affected by shear stress exposure, we measured the association of this integrin with its adaptor protein Shc (fig. 3a). After PAEC exposure to shear stress, the 46- and 52-kDa Shc isoforms showed a significantly higher association with integrin $\alpha_v\beta_3$ than static controls (shear: 46 kDa, 1.6 ± 0.3-fold; 52 kDa, 1.8 ± 0.3-fold). The specific elastase inhibitor MEO significantly reduced the interaction of the integrin and the Shc isoform of 52 kDa (shear + MEO: 46 kDa, 1.3 ± 0.3-fold; 52 kDa, 1.3 ± 0.2-fold over the static controls; fig. 3b).

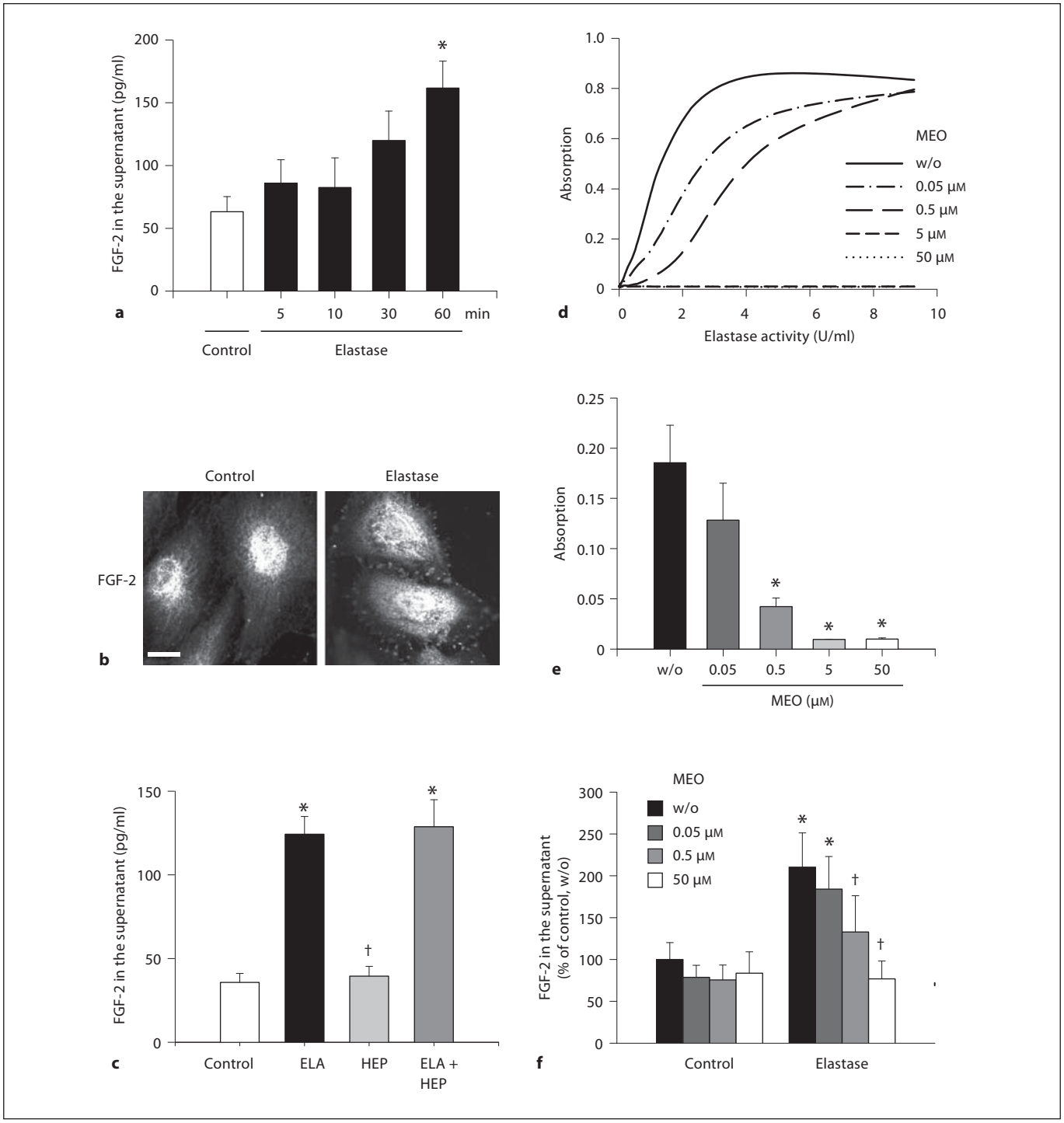

Fig. 4. Elastase induces the release of FGF-2. **a** PAEC were stimulated with porcine pancreatic elastase (0.5 U/ml). Supernatants were collected for quantitative analysis of FGF-2 (n = 6). * $p < 0.05$ vs. control. **b** FGF-2 immunofluorescence staining of untreated or elastase-stimulated PAEC show a similar translocation as observed under shear stress (2 h, n = 3). Scale bar = 10 µm. ×63. **c** PAEC were left untreated or stimulated with porcine pancreatic elastase (ELA, 0.5 U/ml), heparinase (HEP, 0.5 U/ml) or both (ELA + HEP) for 60 min. Supernatants were analyzed for FGF-2 levels (n = 12 and n = 6 for ELA + HEP). * $p < 0.05$ vs. control; † $p < 0.05$ vs. ELA. The inhibitory effect of different MEO concentrations on various elastase concentrations (n = 3, representative graphs are shown) (**d**) or 0.5 U/ml elastase (n = 3, * $p < 0.05$ vs. w/o) (**e**) correlated with its effect on elastase (0.5 U/ml)-induced FGF-2 release (n = 5) (**f**). * $p < 0.05$ vs. control; † $p < 0.05$ vs. elastase w/o.

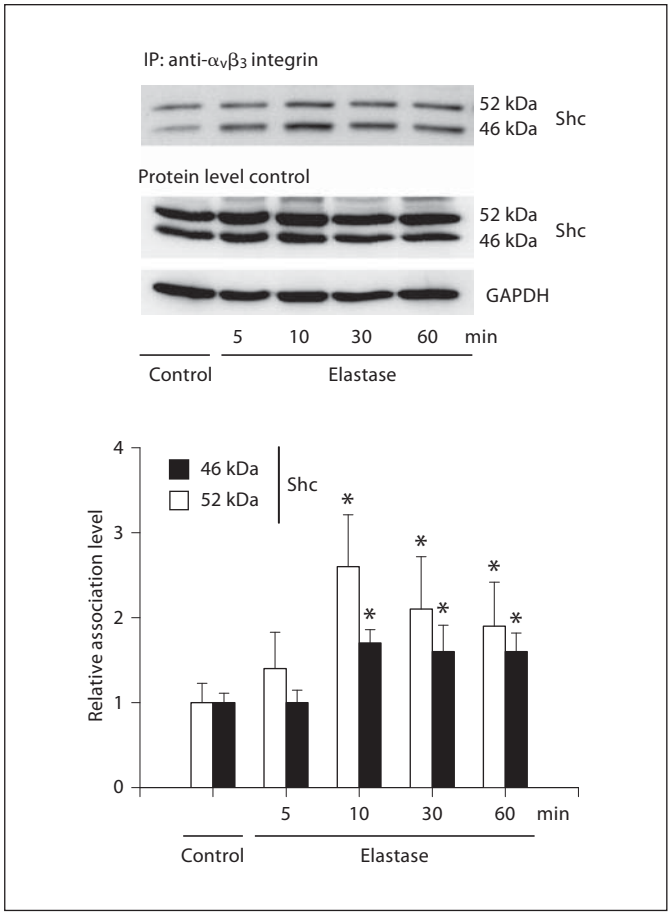

Fig. 5. Elastase increases the association between Shc and the integrin $\alpha_v\beta_3$. PAEC were time-dependently stimulated with elastase (0.5 U/ml). Lysates were subjected to immunoprecipitation (IP) with an $\alpha_v\beta_3$ antibody and immunoblotted against Shc (n = 3). * $p < 0.05$ vs. control. Same lysates were separately immunoblotted for Shc and GAPDH to check equal protein loading.

Shear Stress-Induced p38 MAPK Activation Is Blocked by Elastase Inhibition

Shear stress application to PAEC resulted in an enhanced phosphorylation of p38 MAPK and ERK1/2 (fig. 3c). The inhibition of elastase with both the specific inhibitor MEO as well as the unspecific inhibitor CHY prevented the shear stress-induced p38 MAPK phosphorylation but not ERK1/2 activation. The integrin $\alpha_v\beta_3$ blocker ABC also reduced the activation of p38 MAPK (fig. 3d). In contrast, the phosphorylation of ERK1/2 was not reduced after inhibition of elastase or the integrin $\alpha_v\beta_3$ in cells exposed to shear stress. Neither of the inhibitors affected p38 MAPK and ERK1/2 phosphorylation levels in static control cells.

Effects of External Elastase on Static Cells

Elastase Treatment Induces an Increase in FGF-2 in the Supernatant

Since the data obtained in this study suggest that an endogenous, as yet not molecularly characterized elastase plays a causal role in the shear stress-induced increase in the extracellular concentration of FGF-2, we further investigated whether external elastase treatment could mimic this effect in non-shear-stressed cells. For this purpose, static PAEC were treated with 0.5 U/ml of porcine pancreatic elastase. Application of elastase for 5, 10, 30 or 60 min increased the concentration of FGF-2 in the supernatant in a time-dependent manner, reaching a significant difference after 60 min [control 63.2 ± 12.0 pg/ml, elastase (60 min) 161.7 ± 21.4 pg/ml; fig. 4a]. Immunofluorescence revealed a similar translocation of FGF-2 to the cell membrane as observed under shear stress (fig. 4b). As measured by flow cytometry, elastase did not induce FGF-2 release from endothelial cells kept in suspension: whereas there was a significant translocation of FGF-2 to the cell surface in cells attached to their matrix by 50% relative to control values (control 1.00 ± 0.05 vs. elastase 1.50 ± 0.15, n = 13, p = 0.005), the same treatment was without effect in cells kept in suspension (control 1.00 ± 0.10 vs. elastase 1.00 ± 0.07, n = 7).

In contrast to the treatment with elastase, treatment with heparinase I (0.5 U/ml) for 60 min did not alter the concentration of FGF-2 in the supernatant (control 35.8 ± 5.3 pg/ml vs. heparinase 39.5 ± 5.8 pg/ml). Heparinase I cleaves heparin and heparan sulfates on the cell membrane, known to be extracellular binding sites for FGF-2 [23]. Simultaneous application of elastase and heparinase did not further increase the FGF-2 concentration in the supernatant when compared to elastase treatment alone (elastase 124.3 ± 10.6 pg/ml vs. elastase + heparinase 128.7 ± 16.2 pg/ml; fig. 4c).

MEO inhibited the activity of pancreatic elastase in a concentration-dependent manner. Total inhibition of elastase activity was achieved using 5 or 50 μM of the inhibitor (fig. 4d, e). The inhibitory effect of different concentrations of MEO on the elastase activity correlated well with its inhibitory effects on FGF-2 release (fig. 4f).

Elastase Treatment Activates the Integrin $\alpha_v\beta_3$

Similar to our findings with shear stress, treatment with porcine pancreatic elastase induced an enhanced interaction of the integrin $\alpha_v\beta_3$ with Shc, as revealed by co-immunoprecipitation. Already 10 min after the onset of elastase treatment a significantly higher amount

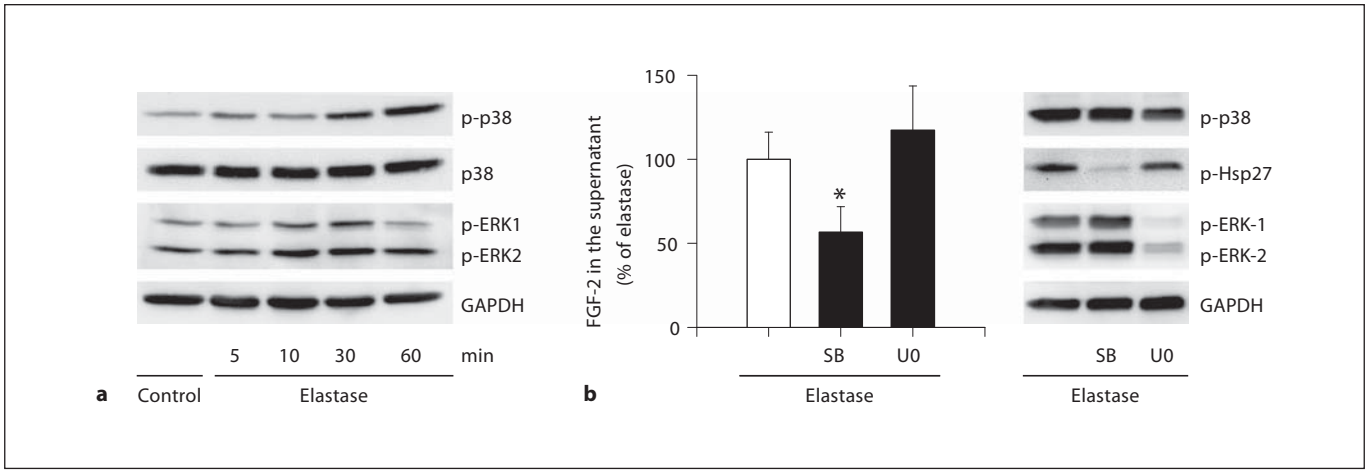

Fig. 6. Elastase causes activation of p38 MAPK and ERK. P38 MAPK inhibition decreases elastase-induced FGF-2 release. **a** Treatment with porcine pancreatic elastase activates p38 MAPK (n = 6) and ERK (n = 6). Equal protein loading was confirmed with total p38 MAPK and GAPDH. **b** PAEC were treated with the p38 MAPK inhibitor SB202190 (SB) or the MEK1/2 inhibitor U0126 (U0) (10 μM each). Cell supernatants were analyzed for FGF-2 release using an ELISA (n = 12, ANOVA RM on ranks, Student-Newman-Keuls) * $p < 0.05$ vs. elastase. Immunoblotting of cell lysates was performed to confirm inhibitory effects of the pharmacological substances used. Since SB202190 does not block p38 MAPK phosphorylation directly, the activation of the downstream target protein Hsp27 was studied instead. Equal protein loading was controlled with GAPDH.

of the Shc isoforms was associated with the integrin [elastase (10 min): 46 kDa, 2.6 ± 0.6-fold; 52 kDa, 1.7 ± 0.2-fold over controls]. This interaction was maintained over 60 min of elastase treatment [elastase (60 min): 46 kDa, 1.9 ± 0.5-fold; 52 kDa, 1.6 ± 0.2-fold over controls; fig. 5].

Pharmacological Inhibition of p38 MAPK Blocks Elastase-Induced FGF-2 Release

To analyze whether elastase treatment activated MAP kinases in a similar manner as shear stress, p38 MAPK and ERK activation was analyzed. Application of porcine pancreatic elastase led to an increased phosphorylation of both p38 MAPK and ERK in a time-dependent manner (fig. 6a).

To study the impact of MAP kinase activation on the elastase-induced FGF-2 release, cells were treated with the specific inhibitors SB202190 (for p38 MAPK) or U0126 (for MEK1/2). The effective inhibition was monitored by Western blot analysis. The effect of SB202190 was examined by analyzing the phosphorylation of Hsp27, a downstream target of p38. Whereas p38 MAPK inhibition significantly reduced the FGF-2 increase in the supernatant (to 57 ± 15%), ERK inhibition had no influence on the elastase-induced release of this growth factor (fig. 6b).

Discussion

The main finding of this study is that shear stress induces integrin activation and FGF-2 release partly via a novel protease-dependent pathway. Due to its design, which focused on the comparability with our own earlier study [22] and others [24–26], the experiments cannot answer the question whether this protease activation required a minimum level of shear stress or whether the protease release would have gradually changed with different levels of shear stress.

However, our results clearly show that exposure of resting endothelial cells to arterial levels of laminar shear stress results in an enhanced enzymatic activity in their supernatant that can be characterized as an elastase. An increased concentration of this elastase was already measurable after 5 min of shear stress application, indicating that the endothelium is able to respond very quickly to increased levels of flow. The enzyme is, however, different from pancreatic and neutrophil elastases and other proteases with elastolytic activity. This elastase mediates the shear stress-induced activation of the integrin $\alpha_v\beta_3$ and subsequently of the p38 MAPK pathway, leading to the release of FGF-2. Furthermore, we were able to demonstrate that treatment of PAEC with exogenously applied porcine pancreatic elastase induced virtually the same ef-

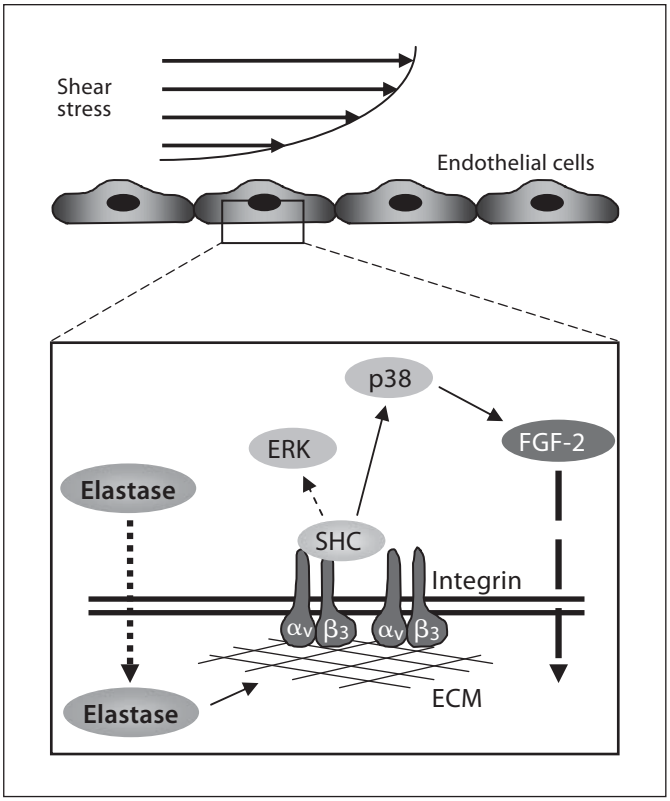

Fig. 7. Proposed model for shear stress-induced FGF-2 release from endothelial cells. Shear stress induces the release of an endothelial elastase, activating integrin $\alpha_v\beta_3$ outside-in signaling and thereby intracellular signaling cascades. FGF-2 is released via p38 MAPK.

fects as observed under shear stress. The present study extends previous work from our laboratory demonstrating that shear stress induces the release of FGF-2 from endothelial cells [22]. This process was found to be specifically dependent on the integrin $\alpha_v\beta_3$ but the connection between shear stress and integrin activation remained unclear.

The endothelial FGF-2 release was reduced by the elastase inhibitor MEO which is specific for enzymes of the elastase family [27]. Moreover, the substrate MeOSuc-Ala-Ala-Pro-Val-pNA, also considered to be specific for neutrophil and pancreatic elastase, was cleaved by this enzyme [28, 29]. The presence of enzymes which have an elastolytic activity in intact vessels has been reported previously: Hornebeck et al. [30] isolated an elastase from pig aortas that exhibited an elastolytic activity similar to pancreatic elastase. It is unlikely, however, that the enzyme is identical with neutrophil and pancreatic elastase, since we found no transcripts of these elastases in our cells. Of note, the specific substrate is not cleaved by other proteases, like cathepsins or MMPs, which previously also have been found to be activated, released or inhibited after shear stress exposure [15, 16]. Thus, the involvement of proteases from the cathepsin and MMP family can be excluded in the present setting. Although some of these proteases also cleave elastin and can therefore be termed elastolytic enzymes, the activity of the endothelial enzyme which we detected was comparable solely to neutrophil and pancreatic elastase because they, in contrast to the other elastolytic enzymes, degrade the same specific substrate. Therefore, we propose that this endothelial serine protease with true elastase activity is a not yet described mediator of endothelial shear stress responses. The major evidence for this conclusion is the following: (1) susceptibility to specific elastase inhibitors, (2) cleavage of the specific elastase substrate and (3) appropriate molecular weight.

The characterization of the enzyme as an elastase based on the specific substrate does not mean that elastin is the only matrix protein targeted by the enzyme. Neutrophil elastase, for example, has a broad spectrum of matrix substrates such as collagens, fibronectin, laminin and proteoglycans, and also cleaves some cytokines [31, 32]. Further studies are required to identify the matrix effects of the endothelial elastase in more detail and also to clarify whether its activity in the intact vessel wall is further controlled by serine protease inhibitors. Therefore, the mechanism of action of the elastase needs further characterization. Presently, it is unknown whether the elastase is acutely released in its active form from the endothelium in response to shear stress or whether it is activated by another enzyme or signal. Since shear stress still increased elastase activity when integrin $\alpha_v\beta_3$ was blocked, we can exclude that the elastase release is preferentially due to a direct activation of the integrin $\alpha_v\beta_3$ by shear forces.

As we were currently not able to identify the exact nature of the endothelial elastase which also precluded silencing of the respective gene, we sought to support our conclusions by comparing the effects of shear stress with those in response to exogenously applied pancreatic elastase. Although this enzyme most likely does not possess an identical substrate spectrum, the consistency of the findings was remarkable. Both treatments induced activation of the integrin $\alpha_v\beta_3$ and p38 MAPK and, as a consequence, increased FGF-2 levels in the supernatant. A similar pancreatic elastase-mediated release of FGF-2 has previously been described in cultures

of pulmonary fibroblasts. Based on the correlation of FGF-2 release with the liberation of sulfated glycosaminoglycans, known to be extracellular binding sites for FGF-2, the authors concluded that FGF-2 was released by proteolytic digestion from proteoglycan storage sites in the ECM [33, 34]. In another study, cultured pulmonary smooth muscle cells treated with neutrophil elastase also released FGF-2 from ECM storage sites [35]. In contrast, our previous studies have shown that the shear stress-induced increase in extracellular FGF-2 corresponds with a reduction in intracellular FGF-2 [22]. Moreover, we demonstrated that the increase in FGF-2 is critically dependent on activation of the integrin $\alpha_v\beta_3$ and requires the activation of p38 MAPK. The need of these steps can hardly be explained by the 'mere' enzymatic liberation of matrix-bound FGF-2 in our experiments. At present it is difficult to reconcile these apparently contradictory findings. It is possible, however, that the amount of bound FGF-2 is much higher in the ECM and on the surface of pulmonary fibroblasts and smooth muscle cells than in endothelial cells, especially since the fibroblasts were cultivated for 10–12 days [33]. In this case, an additional release from the cells may be less significant as observed in the present study. In our experiments, the cells were seeded on pure matrices or even plastic so that the only possible source for FGF-2 could have been the endothelial cells. Moreover, no extracellular FGF-2 was detected outside the cells by immunohistochemistry after 1 day of culture. Furthermore, since heparinase I, known to release extracellularly bound FGF-2 by inhibiting FGF-2 binding to heparan sulfate proteoglycans [36], failed to increase the FGF-2 concentration in the supernatant in our study, it is likely that matrix binding in our cell cultures was relatively low.

The current study demonstrates that shear stress induces the activation of the integrin $\alpha_v\beta_3$ as assessed by its enhanced interaction with the adaptor protein Shc. This was reduced when the cells were pre-incubated with the specific elastase inhibitor, indicating that elastase is a critical mediator of the activation. Of note, the exogenously applied pancreatic elastase induced a similar interaction of integrin $\alpha_v\beta_3$ and Shc. Jalali et al. [25] have previously demonstrated that shear stress increases the association of the integrin $\alpha_v\beta_3$ and Shc in endothelial cells. Likewise, Tzima et al. [26] could show that shear stress activates the integrin $\alpha_v\beta_3$ in endothelial cells using an antibody that selectively detects the activated integrin $\alpha_v\beta_3$. Our studies now provide a mechanism of shear stress-induced integrin activation that is independent of a direct mechanical activation by shear forces.

Although it has been shown that platelet integrins may be activated by direct cleavage by elastase [37], this is unlikely to have occurred in our study. Firstly, we did not find any change in the size of the integrin α_v-subunit after exposure to pancreatic elastase and, secondly, elastase did not induce FGF-2 release from endothelial cells kept in suspension without any contact with the matrix. This is in agreement with a previous report stating that endothelial cells must be anchored to the matrix in order to record and quantify cellular responses to mechanical strain [38]. Thus, we suggest an elastase-induced remodeling of the ECM architecture as the activating mechanism. Elastase-induced changes in the structure and rigidity of the matrix is likely to enable new (or re-) clustering of integrins into focal adhesion points, a model which has been proposed previously [39]. Alternatively, proteolytic activities may expose hidden protein sequences or even release matrix fragments that have integrin-activating properties [40, 41]. Previous investigations indicate that proteases are able to generate signals by remodeling the ECM [42–44].

Our results further demonstrate that p38 MAPK and ERK were activated by shear stress exposure. Elastase inhibition reduced the shear stress-induced p38 MAPK activation. Likewise, we were able to show that elastase treatment also induced activation of p38 MAPK and ERK. Moreover, we demonstrated that p38 MAPK but not ERK inhibition reduced the elastase-induced FGF-2 release. Therefore, p38 MAPK seems to play a role in shear stress-induced FGF-2 release.

Since several groups have demonstrated that inhibition of the integrin $\alpha_v\beta_3$ blocked the activation of p38 MAPK [45, 46], it is reasonable to argue that flow-induced MAPK phosphorylation is mediated via this integrin. This is in agreement with results reported by Chen et al. [47] showing that direct inhibition of α_v integrins or downregulation of their expression decreased the active level of p38 MAPK. The precise signaling pathway connecting the integrin and MAP kinase activation is still elusive in our setting. However, since shear stress and elastase stimulation induced the association of the integrin with its adaptor protein Shc, downstream signaling may be transduced via Shc and focal adhesion signaling.

In conclusion, the present results support a new model for the translation of mechanical stress into biochemical signals via a novel endothelial elastase (fig. 7). Since FGF-2 is an important growth factor in vascular develop-

ment and maintenance, this model could have profound implications for our understanding of mechanically induced vascular remodeling processes. The precise mechanisms by which elastase activates the integrin $\alpha_v\beta_3$ and downstream signaling will be the focus of further investigations.

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