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Thrombin Induced Secretion of Macrophage Migration Inhibitory Factor (MIF) and its Effect on Nuclear Signaling in Endothelium

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ABSTRACT

The procoagulant thrombin stimulates endothelial cells (EC) to undergo rapid cytoskeleton changes via signaling pathways that induce multiple phenotypic changes, including alterations in permeability, vasomotor tone, adhesion molecule synthesis, and leukocyte trafficking. We studied a novel role of thrombin's action on the endothelium that results in MIF secretion, which is linked to myosin light chain (MLC) and extracellular signal-regulated kinase (ERK½)-dependent nuclear signaling. In bovine pulmonary artery EC (BPAEC), thrombin treatment induced intracellular MLC phosphorylation within 15 min, followed by a significant increase in MIF secretion within 30 min. Thrombin treatment induced biphasic ERK½ phosphorylation with an early phase occurring at 15 min and a later phase at 120 min. To understand the role of MIF secretion in thrombin-induced biphasic activation of ERK½, BPAE cells were treated with (i) recombinant MIF, and (ii) the medium collected from thrombin-treated BPAE cells. These studies demonstrated a sustained monophasic ERK½ phosphorylation. Inhibition of MIF secretion by MIF siRNA or antisense-MIF treatment, along with a neutralizing antibody, attenuated the thrombin-induced second phase ERK phosphorylation, suggesting a direct involvement of MIF in the second phase of ERK½ activation. Pretreatment of BPAE cells with an ERK kinase inhibitor and with antisense-MIF significantly inhibited thrombin-induced nuclear factor kappa (NF-B) activation. These results indicate that MIF secretion and ERK phosphorylation both play a necessary role in thrombin induced NF-B activation. J. Cell. Biochem. 105: 1279–1288, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: MIF-MLCK; THROMBIN-ERK; NFB

Thrombin, a multifunctional serine protease, is generated through activation of the coagulation cascade and stimulates the endothelium to synthesize several adhesion molecules, phosphoinositides, and prostaglandins, as well as stimulates secretion of Von Willebrand factor and tissue plasminogen activator [Lum et al., 1992; Dudek and Garcia, 2001; Kataoka et al., 2003; Klarenbach et al., 2003]. Thrombin mediates its cellular responses through activation of its receptors, a family of protease-activated receptors known as PARs [Coughlin, 1999; O'Brien et al., 2000]. Following tissue injury, thrombin converts soluble plasma fibrinogen into an insoluble fibrin clot, and promotes platelet aggregation [Coughlin, 1999]. In addition to these pro-coagulant effects, thrombin plays a major role in inflammation and the repair of injured tissues [O'Brien et al., 2000]. The thrombin-stimulated endothelium undergoes many phenotypic changes, including increased intercellular gap formation and permeability, changes in cell shape, adhesion molecule synthesis to stimulate recruitment and migration of leukocytes, [Garcia et al., 1995; Mclaughlin et al., 2005]. Although many of these changes are mediated through the induction of new gene expression, specific intracellular pathways are responsible for transcriptional regulation [Ellis et al., 1999].

Recent studies have demonstrated the importance of thrombin signaling in transcriptional control [Chandrasekharan et al., 2004;

Abbreviations used: BPAEC, bovine pulmonary artery endothelial cells; CA, constitutively active; CM, conditioned medium; ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MIF, macrophage migration inhibitory factor; MLC, myosin light chain; MLCK, myosin light chain kinase; PKC, protein kinase C; Tricine, N-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine.

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Minami et al., 2004b]. Treatment of endothelial cells with thrombin induces the expression of multiple transcription factors, including EGR1, FosB, jun B-like immediate early genes, as well as ATF3, MEF2, and NF-B like known transactivators [Kaur et al., 2001; Minami et al., 2004a]. These factors are involved in the regulation of E-selectin [Kaplanski et al., 1997], V-CAM-1 [Minami and Aird, 2001], platelet activating factor-1 [Prescott et al., 1984], and several other proteins involved in endothelial cell activation [Minami et al., 2006]. Thrombin treatment of endothelial cells shown cause a rapid up-regulation of the Down syndrome critical region (DSCR)-1 gene, a negative feedback regulator of calcium-calcineurin-NF-AT signaling [Minami et al., 2006].

In various endothelial cells, a significant induction of macrophage migration inhibitory factor (MIF) protein expression was observed following treatment with thrombin, suggesting a potential role for MIF in thrombin action [Shimizu et al., 2004; Wadgaonkar et al., 2005a]. Involvement of thrombin-dependent signaling is a contributing factor in angiogenesis, atherosclerosis, rheumatoid arthritis, and other diseases where MIF expression is evoked, which suggest a correlation between thrombin pathways and MIF-dependent signaling. Recently, a type II transmembrane protein receptor for MIF, CD74, was cloned through expression cloning and functional analysis [Leng et al., 2003] with high-affinity binding for extracellular MIF [Leng et al., 2003]. The physiological mechanism controlling activation of MIF synthesis, secretion, and binding with this newly discovered receptor is not well described. MIF has been shown to bind to the extracellular domain of CD74, that initiates MIF-dependent signaling of ERK MAP kinase, prostaglandin E2 (PGE_2) production, and cell replication. A receptor-independent mechanism by which MIF may carry out its cellular actions was recently proposed by Kleemann et al. [2000]. In their model, they proposed that, after internalization, MIF interacts with cytosolic c-*Jun*-activating binding protein (Jab1) that modulates the AP1 signaling pathway [Lue et al., 2006]. Recent studies by Duan and Cannon [2000] have correlated MIF overexpression with NF-B activation and inhibition of glucocorticoid response.

Previously, MIF action was correlated with induction of the proliferation-associated gene (PAG-1) [Amin et al., 2003], c-Src tyrosine kinase [Takahashi et al., 1998], intracellular calcium [Fan et al., 2005], and nitric oxide [Amin et al., 2006]. Also, there is evidence suggesting that MIF is a strong contributor to the activities of at least three transcription factors central to the inflammatory and cell replication processes, namely NF-B, PU.1, and E2F1 [Ren et al., 2004; Zicari et al., 2006]. These transcriptional regulators may represent important distal effectors responsible for several of the aforementioned activities of MIF. Recently, we along with others have shown the induction of MIF expression in endothelial cells following thrombin treatment, and its interaction with myosin light chain kinase (MLCK) [Shimizu et al., 2004; Wadgaonkar et al., 2005a]. Since MIF is induced by thrombin and appears to be involved in AP1 and NF-B activation, it will be important to study the MIF-MLCK interaction in greater detail to correlate cytoskeletal rearrangement and thrombin-induced nuclear signaling [Kleemann et al., 2000; Wadgaonkar et al., 2005b].

Based on thrombin's known signaling pathways and its induced proliferative effects on various cell types including endothelial cells [Petrenko and Moll, 2005] coupled with results showing the induction of MIF secretion from pulmonary endothelial cells [Wadgaonkar et al., 2005a], we hypothesized that MIF secretion and MIF-dependent signaling play an important role in nuclear transcription. In this paper we demonstrate that thrombin stimulates endothelial cell MIF secretion, that correlates with ERK phosphorylation- and NF-B-dependent transcriptional regulation. Our results address the potential for novel interactions between coagulation proteases, in concert with MIF, for generating nuclear signaling required for thrombin action.

MATERIALS AND METHODS

ENDOTHELIAL CELL CULTURES AND REAGENTS

Bovine pulmonary artery endothelial cells (BPAEC) were obtained frozen at 16 passages from American Type Culture Collection (CCL 209; Manassas, VA) and were utilized at passages 19–24 and cultured in MCDB medium (Biofluids, Rockville, MD) containing 2 mM sodium bicarbonate (pH 7.3), 2 mM glutamine, non-essential amino acids, 2 U/ml heparin, 1 mg/ml hydrocortisone, 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 15 mg/ml endothelial cell growth supplement, and 1% antibiotics (penicillin, streptomycin, Fungizone) and as described earlier [Borbiev et al., 2003]. BPAECs were cultured in complete media and maintained at 37°C in a humidified atmosphere of 5% CO_2-95% air, and were grown to contact-inhibited monolayers with the typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin and resuspended in fresh culture medium, then passaged to the appropriate sized flasks or dishes. Bovine thrombin was obtained from Sigma–Aldrich (St. Louis, MO) and used at 100 nM concentration. Unless specified, reagents were obtained from Sigma–Aldrich. MIF monoclonal and polyclonal antibodies against human MIF were obtained from R and D Systems (MN). Myosin light chain di-phospho antibody, which recognizes phosphorylated Ser^{19} and Thr^{18} of MLC, was used as previously described [Wadgaonkar et al., 2005b]. Cyclohexamide dissolved in 0.2 M Dimethyl Sulfoxide (DMSO) at 100 mg/ml was purchased from Sigma–Aldrich and used at 10 g/ml concentration for BPAEC treatment. As a solvent control 0.2 nM DMSO was used for BPAEC treatment. MLCK specific inhibitor ML7-hydrochloride was purchased from (Sigma-Aldrich), dissolved in DMSO and 50% ethanol at 10 mM stock solution and used at 100 nM concentration. As a solvent control DMSO-ethanol was used at appropriate fold dilution in tissue culture medium with serum and growth factors. MAP kinase inhibitor PD 98059 was purchased from Sigma–Aldrich, dissolved in DMSO at the concentration of 50 mM and used at 25 M concentration.

Recombinant human MIF protein was purchased from R and D Systems (MN). This protein has a 94% homology with bovine MIF at the amino acid level. The recombinant protein was tested for its endotoxin content, which was found to be <1.0 EU per 1 g of the cytokine as determined by the LAL method. This MIF protein induced myosin light chain phosphorylation as well as time dependent MAP kinase activation, suggesting that it is a biologically active protein.

TRANSFECTION AND LUCIFERASE ASSAY

Endothelial cells (EC) were seeded (2×10^5 cells/well) in six-well plates and were transfected using the Fugene transfection method (Roche, NJ) with a total of 6 g of DNA/plate and transfected in triplicate. The EC were co-transfected with a luciferase reporter gene construct (1 g/well), regulated by either an E-selectin promoter or by five copies of the consensus sequence of NF-B DNA binding site [(NFkB) 5-Luc] (Stratagene). Cells were also co-transfected with a cytomegalovirus early promoter driven beta-galactosidase gene (CMV bgal) and the data was normalized to the activity of beta-galactosidase activity. Cell lysates were prepared for luciferase and -galactosidase (gal) activity assays as per manufacturer's instructions 24–48 h after transfection (Promega, Madison, WI).

SILENCING OF MIF EXPRESSION

MIF silencing RNA oligos were selected from the possible four silencing regions identified by the Ambion program and synthesized from Ambion, Inc. As a control, a scrambled oligo containing identical nucleotides to MIF SiRNA was used. BPAEC cells were plated in gelatin-coated 12-well plates and grown in BPAEC growth medium as described earlier [Borbiev et al., 2003; Wadgaonkar et al., 2005b]. Conditions for transient transfections of plasmids and silencing RNA in BPAE cells were established using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and the GenePORTER 2 transfection reagent (GTS, San Diego, CA). GenePORTER-dependent transfections were done as follows: cells were plated at 60–70% confluence. The next morning, cells were rinsed twice with phosphate-buffered saline and 900 l of serum-free medium was added to each well and incubated at 37°C. To prepare the siRNA complexes, silencing RNA for green fluorescence protein (GFP) or MIF was diluted in buffer B (Gene PORTER, composition not described, patented by GTS) at concentrations of 50 and 100 M and incubated at room temperature for 5 min. Diluted oligos were added to the diluted GenePORTER 2 reagent, then mixed and incubated for 10 min at room temperature. This mixture was directly added to the cells in the serum-free medium. After 4 h of transfection, growth media was added without removing the transfection reagent. 12 h after transfection, cells were supplemented with complete growth medium for another 36 h.

REAL TIME AND REVERSE TRANSCRIPTASE (RT) PCR

Total RNA from control and thrombin treated samples was isolated using Trizol (Invitrogen). Total RNA (1 g) was used for reverse transcriptase reaction using SuperScript First-Strand Synthesis system. For MIF PCR the Sybergreen method was used. The primers for bovine MIF amplification were designed from the Sigma Genosys system. The sequence of forward primer used was 5′ CTCTCCGAGCTCACCCAGCAG-3′ (forward) and reverse primer sequence was 5′-CGCGTTCATGTCGTAATAGTT-3' (reverse). As internal control, GAPDH or 18S rRNA primers and probes were purchased from (Sigma Genosys).

ADENOVIRUS VECTOR PREPARATION AND EXPRESSION OF SENSE AND ANTISENSE GFP-MIF

The recombinant Adv-sense and anti-sense GFPMIF (AdV-GFPMIF) adenoviruses were constructed using Adeasy-adenovirus expression system as described earlier [Petrache et al., 2003]. Briefly, full length human MIF cDNA obtained from a cDNA expression library was amplified by primers that contained the restriction enzyme then was synthesized and cloned into a pAdTrack-CMV shuttle vector in the sense and antisense orientation. After this plasmid was confirmed and purified, it was cut with *Pme*I and transformed into BJ5183 containing pAdeasy1. Recombinant viruses were isolated on kanamycin plates and verified by restriction analysis. A recombinant clone was then cut with *Pac*I and transfected with Lipofectamine (according to manufacturer's protocol) into human embryonic kidney (HEK)-293 cells. After 10 days, cell lysate was prepared and used to infect more HEK-293 cells. The presence of infectious virus was confirmed by green fluorescent protein (GFP) fluorescence.

ELISA ASSAY

For quantitative measurement of MIF, *ChemiKine*™ Human Macrophage Migration Inhibitory Factor (MIF) Kit was used as described earlier by our group [Gao et al., 2007]. This is a "sandwich" enzyme immunoassay (EIA), which can measure natural and recombinant forms of the migration inhibitory factor (MIF). In this assay system, pre-coated mouse monoclonal antibody generated against human MIF was used to capture human MIF in a sample. This antibody was also capable of detecting bovine MIF. Simultaneously, HRP-labeled MIF- specific mouse monoclonal antibody was used to detect MIF in the sample. With the addition of the Color Generating Solution, the amount of MIF was detected. The standard curve demonstrated a direct relationship between optical density (OD) and MIF concentration: "i.e., higher the OD, higher was the MIF concentration in the sample".

IMMUNOPRECIPITATION/WESTERN BLOT

Whole endothelial cell extracts were prepared by mechanical disruption in 500 l of lysis buffer (100 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 g/ml leupeptin, 1 g/ml aprotinin, 1.5 g/ml pepstatin A, 0.2 mM levamisole, 10 mM beta-glycerophosphate, and 0.5 mM benzamidine) per 100-mm^2 plate of confluent cells. Particulate matter was removed by centrifugation at 10,000*g* for 20 min. Extracts were pre-cleared with rabbit or mouse secondary antibody and IgG/IgA-agarose beads. Supernatants were incubated with 10 g/ml MLCK antibody (K36: Sigma–Aldrich) or MIF antibodies (R&D), respectively. The antibodies were then bound to 50 l of IgG/IgA-agarose beads. These beads were washed three times with 1 ml of lysis buffer each time and resuspended in SDS sample buffer. The eluted proteins were boiled for 3 min and separated by SDS–polyacrylamide gel electrophoresis (6–10%). The separated proteins were transferred to nitrocellulose membranes, and blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.5% Tween-20) for 3 h at room temperature. The blots were then washed three times in TBST buffer, incubated with a donkey anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech), and again

washed three times in TBST. Bound proteins were visualized using the ECL chemiluminescence reagent (Amersham Pharmacia Biotech) followed by autoradiography for 30 s to 60 min.

STATISTICAL ANALYSIS

Data in the figures are presented as the mean ± SE of one representative experiment. Differences between the various treatments were tested by Student's *t*-test and were considered statistically significant at $P < 0.05$.

RESULTS

THROMBIN-INDUCES BIPHASIC ERK ACTIVATION IN BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS

We have previously shown that the thrombin-induced contractile events, gap formation, and barrier dysfunction, occur via MLCK-dependent mechanisms [Borbiev et al., 2003]. In addition, we have identified the functional interaction between MIF and MLCK in endothelial cells [Wadgaonkar et al., 2005a], and noted that MLCK activation in the endothelium contributes to NF-B dependent signaling [Wadgaonkar et al., 2005b]. To further explore the functional consequences of the MIF-MLCK interaction, we examined signaling pathways that couple the thrombin receptor with inducible translocation of NF-B. We have previously shown that thrombin potently increases intracellular Ca^{2+} and activity of the MAP kinases, including ERK, in pulmonary endothelial cells [Borbiev et al., 2003]. In order to understand the roles of MLCK activation and MIF secretion in downstream signaling, we first examined ERK activation in confluent ECs treated with thrombin (100 nM). We found that thrombin-induced ERK activation was biphasic with rapid time-dependent increases in phospho-ERK immunoreactivity (maximal at 15 min) (Fig. 1A), followed by a downregulation of ERK phosphorylation. Beyond 120 min of thrombin stimulation, sustained ERK phosphorylation was again observed for more than 2–3 h (Fig. 1B). These results suggest that the initial ERK phosphorylation phase and the later phase are most likely involved in generating different signaling pathways.

THROMBIN INDUCED INTRACELLULAR MIF EXPRESSION AND SECRETION

To further understand MLCK activation and the role of MIF secretion in ERK phosphorylation, we first studied thrombin – induced MLCK dependent MLC-phosphorylation and its effect of MIF expression. We found that after 30 min of thrombin treatment, intracellular MIF protein expression levels were increased three- to fourfold. To further investigate thrombin induced MIF expression and secretion, we monitored mRNA levels of MIF by real time PCR using the Sybergreen method. Quantitative real time PCR analysis, (Fig. 2B) showed that, MIF mRNA levels when compared to the normalized 18S RNA control, were upregulated after thrombin treatment. At 60–90 min after thrombin treatment there was a twofold induction in MIF expression. These results are analogous to earlier studies by Chandrasekharan et al. [2004] and Shimizu et al. [2004], where thrombin induced MIF transcription was measured in endothelial cells. Shimizu et al. [2004], showed that Factor Xa can induce MIF transcription within 30 min and ATIII treatment was able to inhibit

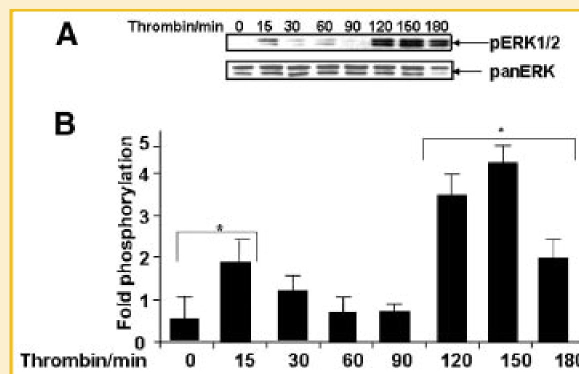

Fig. 1. Effect of thrombin on endothelial ERK½ phosphorylation. A: BPAE cells treated with thrombin (100 nM) for different time points and total extracts were prepared, separated on SDS–PAGE gels and immunoblotted by phopsho-ERK½ and total ERK antibodies. B: Fold induction of ERK½ phosphorylation was plotted from the average of three independent experiments. * represents significant increase in ERK phosphorylation over the control.

FXa induced MIF expression. Our results have shown a more precise quantitative measure of thrombin induced endothelial cell MIF expression and secretion.

We also showed that, thrombin significantly stimulated MIF secretion into the EC culture medium, which was detected after 30 min of thrombin treatment (Fig. 2B,C). To determine the effects of MLCK activation on MIF secretion, cells were pretreated with the MLCK inhibitor ML-7, and then stimulated with thrombin, this resulted in a significant time-dependent inhibition of MIF secretion. As a control 10% ethanol did not show inhibition of thrombin induced MIF secretion. Figure 2A confirms and as per our previous report that thrombin-induced MLCK activation and MLC phosphorylation begins rapidly after PAR1 ligation and peaks at 15–30 min [Garcia et al., 1995]. These results temporally correlated well and suggest a role for thrombin-induced MLCK activation in MIF expression and secretion. We further immuno-precipitated the MIF secreted from thrombin-treated ECs using a MIF polyclonal antibody with detection by monoclonal antibody for Western blotting (Fig. 2C). We detected significant amounts of MIF in the thrombin-treated endothelial culture medium. This response was noted to be time dependent. These results suggest that thrombin-induced MIF secretion is MLCK activation dependent. To further analyze the contribution of preformed versus newly synthesized MIF protein in thrombin induced secretion, new protein synthesis was blocked by cyclohexamide treatment. Thrombin induced MIF secretion was attenuated by cyclohexamide treatment at 60 and 90 min. At earlier time secretion was not significantly inhibited. This suggests that preformed MIF may have been secreted within 30–60 min but new MIF synthesis is required for further secretion after 60 min.

THROMBIN-INDUCED MIF SECRETION IS REQUIRED FOR THE SECOND PHASE OF ERK PHOSPHORYLATION

To understand the role of MIF secretion and MLCK activation in ERK phosphorylation, we first inhibited MIF expression in BPAE cells using an anti-sense MIF adenovirus. This resulted in significant

inhibition of intracellular MIF expression (Fig. 3A). BPAE cells infected with GFP-tagged sense and antisense MIF showed more than 90% transfection efficiency. These cells were analyzed for ERK phosphorylation at 120–150 min (Fig. 3B). Overexpression of intracellular MIF did not significantly increase ERK phosphorylation, however inhibition of MIF expression reduced thrombin-induced ERK phosphorylation in the late phase (Fig. 3B). To further understand the effects of secreted MIF in ERK phosphorylation, BPAE cells were pre-incubated for 60 min with a neutralizing antibody against MIF (Fig. 3C) before thrombin treatment. After thrombin treatment ERK phosphorylation was determined at time points between 15 and 150 min. MIF antibody treatment did not affect ERK phosphorylation at the 15 min time point however, at 150 min ERK phosphorylation was significantly blocked. These results suggest a correlation between thrombin induced MLCK activation and MIF secretion, and also indicate that MIF secretion may be involved in the biphasic response of ERK phosphorylation (Fig. 3B,C).

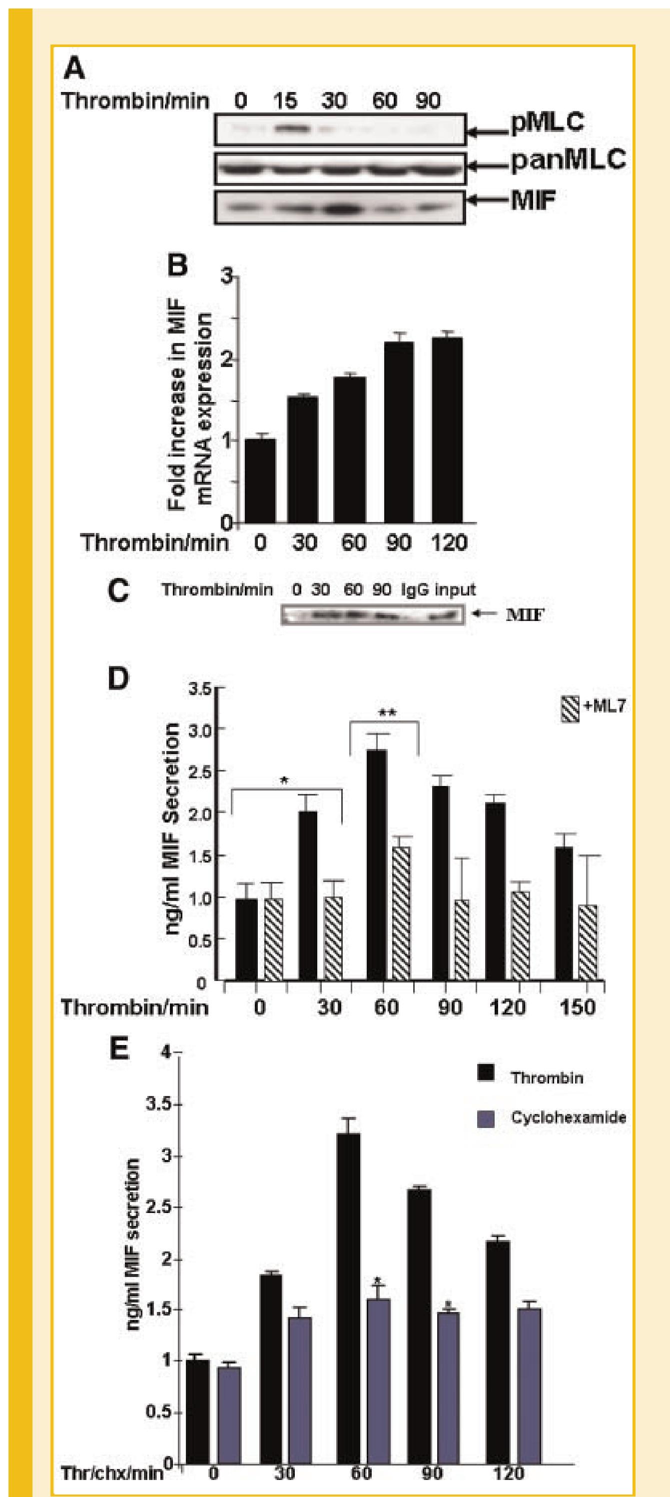

Fig. 2. Thrombin induces time-dependent intracellular MIF expression and secretion. A: BPAE cells were grown to confluence, preincubated in serum free medium and treated with thrombin at different time-points, and culture medium was collected after 0, 30, 60, and 90 min. Total extracts were prepared and blotted for MLC phosphorylation and induced MIF expression was measured. B: Thrmobin induced MIF expression was measured at mRNA level using Sybergreen real time PCR. Total RNA from control and thrombin treated BPAE cells were isolated using Trizol (Invitrogen). Total RNA (1 g) was used for reverse transcriptase reaction using SuperScript First-Strand Synthesis system. C: Endothelial cell culture medium was collected after thrombin treatment, and immuno-precipitated using a MIF-specific polyclonal antibody and Western blotted. MIF protein was identified using a MIF monoclonal antibody. As an input recombinant MIF was immuno-blotted. D: ELISA assay for MIF detection was performed to measure secreted MIF. BPAE cells grown to confluence were pretreated in serum free medium and ML7 (100 nM) or DMSO-ethanol in control samples, then treated with thrombin (100 nM) after 1 h. Culture medium was collected after various time points, and an ELISA assay was performed to detect MIF secretion. Thrombin-induced MIF secretion was attenuated by Myosin light chain kinase inhibitor ML7. * represents significant difference in phosphorylation after thrombin treatment. ** represent significant inhibition of ERK phosphorylation after ML7 treatment. E: Effect of cyclohexamide on thrombin induced MIF secretion. BPAE cells preincubated with cyclohexamide (10 g/ml) containing serum free medium or DMSO control for 1 h and then treated with thrombin for different time points. Culture medium was collected after various time points, and an ELISA assay was performed to detect MIF secretion. Thrombin-induced MIF secretion was significantly attenuated by cyclohexamide treatment at 60–90 min time point. * represents significant difference in MIF secretion after cyclohexamide treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RECOMBINANT MIF INDUCES ERK1/2 PHOSPHORYLATION: ROLE OF MLCK ACTIVATION

In order to confirm our initial findings that increased levels of extracellular MIF may be responsible for the significant increase in ERK phosphorylation following thrombin stimulation, we first studied the effect of secreted MIF in BPAE cells using condition medium collected after 60 min of thrombin treatment. After inactivating thrombin, ERK phosphorylation was monitored. Condition medium collected from thrombin treated cells was sufficient to induce significant ERK phosphorylation within 30 min (Fig. 4A). Next, we treated BPAE monolayer cells with recombinant MIF at different time points, and ERK phosphorylation was again monitored. Within 15 min of MIF (10 ng/ml) treatment, significant ERK phosphorylation was noted (Fig. 4B). This suggested that recombinant MIF is active and sufficient to induce signaling defined by ERK phosphorylation. To study the involvement of MLCK activation in this process, we inhibited MLCK kinase activity (ML7),

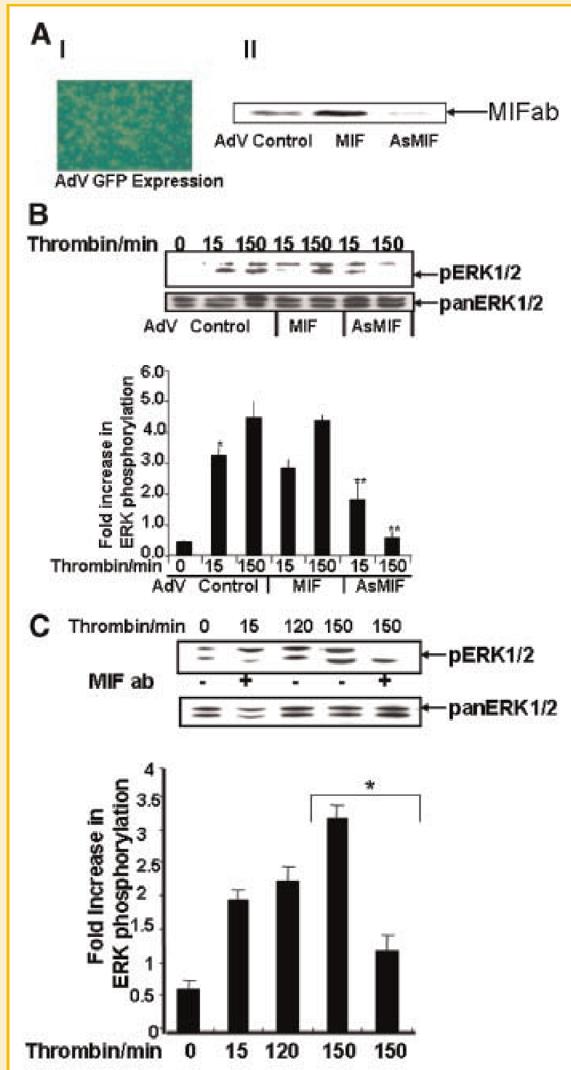

Fig. 3. Inhibition of MIF expression and secretion attenuated thrombin induced ERK phosphorylation. A: BPAE cell monolayers were infected with adenoviruses expressing GFP alone, GFPMIF and MIF antisense orientation and monitored using GFP expression for transfection efficiency. The presence of infectious virus was confirmed by green fluorescent protein (GFP) fluorescence. Green cells represent total transfected cells, >95% cells with GFP expression (I). Twenty four hours post-infection MIF expression was measured by Western blotting for MIF expression (II). B: Adenoviral GFP, GFPMIF, and anti-sense MIF (AsMIF) expressing cells were treated with thrombin, and total extracts were separated on SDS-PAGE and immunoblotted with a phospho-ERK½ antibody. Significant differences between control, thrombin treated (*) and antisense-MIF overexpressing cells in ERK phosphorylation are shown by (**). C: BPAE monolayers before thrombin treatment incubated with neutralizing MIF antibody for 1 h then treated with thrombin. Total extracts were immunoblotted for phospho-ERK antibody. MIF antibody-treated cells showed significant inhibition of thrombin-induced ERK phosphorylation at 150 min. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

and assessed the effects of recombinant MIF in ERK phosphorylation. At early time points, we found significant inhibition of ERK phosphorylation by ML7 (Fig. 4C), whereas at later time points the attenuation of MLCK activation was insufficient to inhibit ERK phosphorylation (Fig. 4C,D). This suggested that MLCK may be

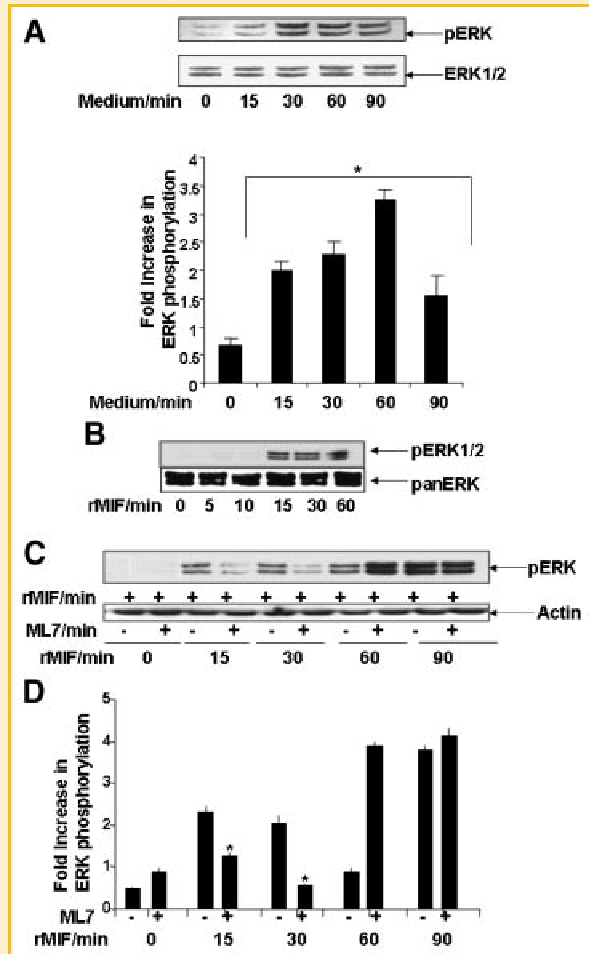

Fig. 4. Recombinant MIF induced endothelial ERK½ phosphorylation and effect of MLCK inhibition. A: Secreted MIF induced ERK½ phosphorylation. Endothelial cells were treated with thrombin 60 min and medium from treated plates was collected to incubate the untreated BPAE cells for various time points. Significant ERK phosphorylation (*) was noted after 60 min of medium treatment. B: BPAE cells were treated with recombinant MIF (10 ng/ml) for different time points, and total extracts were prepared, separated on SDS-PAGE gels, and immunoblotted by phopsho-ERK½ and total ERK antibodies. Significant ERK phosphorylation was induced following recombinant MIF treatment. C,D: Cells were pretreated with ML7 or ethanol as a solvent control for 30 min and stimulated with recombinant MIF for various time points. Significant inhibition of ERK phosphorylation by ML7 was noted at early time points of rMIF treatment.

primarily involved in MIF secretion, and that the sustained ERK phosphorylation response induced by MIF was independent of MLCK activation.

EFFECT OF MIF INHIBITION ON THROMBIN-INDUCED NF-KB ACTIVATION

To characterize the modulatory role of MIF in thrombin-induced signal transduction pathways, we examined the effects of MIF inhibition on NFB transactivation and induced gene expression following thrombin treatment. Initial experiments examined the NFB transactivation function by transfecting a promoter construct containing multiple NFB binding sites driving a luciferase expression vector, as we have

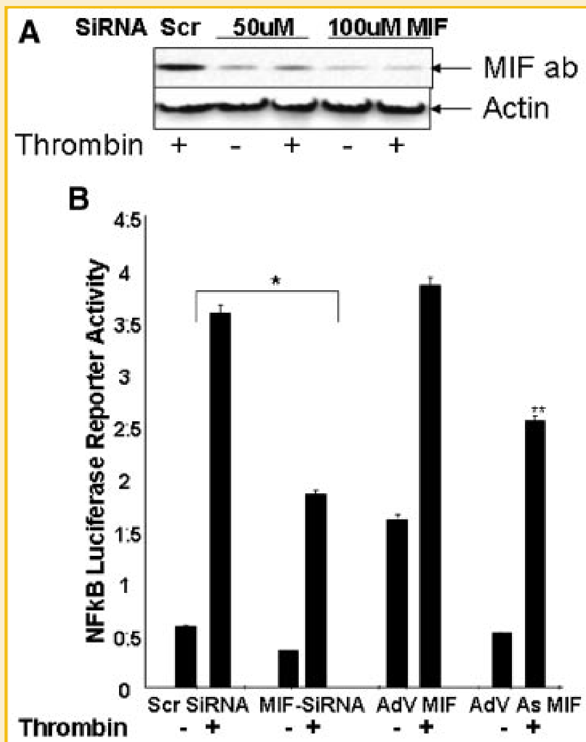

Fig. 5. MIF potentiates thrombin induced NF-B activation. A: siRNA treatment and inhibition of MIF expression. Endothelial cells at 70% confluency were transfected with GenePorter and MIF siRNA at 50 and 100 M concentration as described in Materials and Methods Section. Forty eight hours post-transfection, cells were treated with thrombin for 30 min and immunoblotted for MIF expression. Increasing MIF-siRNA concentration caused significant decreases in MIF expression. B: Endothelial cells transfected with scrambled or MIF siRNA and NF-B luciferase reporter were treated with thrombin 48 h after transfection. Endothelial cells transfected with NF-B Luciferase reporter plasmid were further infected with Adenoviruses expressing sense and anti-sense MIF. Forty eight hours post-transfection, cells were treated with thrombin (100 nM) for 6 h, and total cell extracts were measured for luciferase activity.

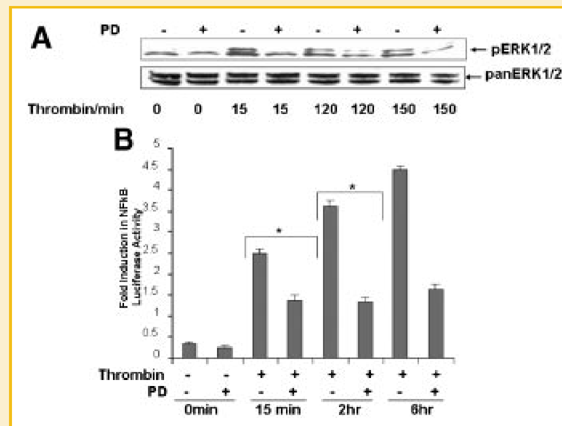

Fig. 6. Contribution of Thrombin and MIF-induced ERK phosphorylation in NF-B transactivation. A: NF-B Luciferase expression vector transfected cells after 48 h were treated with PD98059 at 25 M concentration for 15 min, 120 min, and 6 h. DMSO at appropriate concentration was used as a solvent control. Thrombin treatment started following removal of the PD media from 15 and 120 min PD treatment for total 6 h. In 6 h PD treated cells thrombin was added at the beginning of PD treatment. Total cell extracts were prepared to measure ERK phosphorylation. Cells were harvested after 6 h and analyzed for luciferase activity. B: Endothelial cells 24 h post-transfection were treated with thrombin for 6 h, and total cell extracts were measured for luciferase activity.

previously reported [Wadgaonkar et al., 2005b]. In this transfection assay, we used an oligo-based delivery of siRNA and performed a direct transfection of siRNA oligos into cultured endothelial cells. Using siRNA against GFP, we first optimized the conditions for EC transfection and achieved >90% transfection efficiency using Gene-Porter and Lipofectamine 2000 reagents. Transfected cells were then treated with thrombin, and tested for MIF expression (Fig. 5A). MIF expression was noted to be significantly reduced by 100 M siRNA treatment. Thrombin induced a fivefold increase in luciferase gene expression, which was significantly reduced (50–60%) by silencing of MIF expression via siRNA and anti-sense MIF vectors. These results suggest that MIF expression is required for optimal thrombin-induced NFB transactivation (Fig. 5B).

EFFECT OF THROMBIN INDUCED MIF AND ERK PHOSPHORYLATION ON NF-κB TRANSACTIVATION

In the final experiment, we determined that MIF-dependent activation of ERK and sustained ERK phosphorylation in endothelial cells is also involved in thrombin-mediated NF-B transactivation. For this experiment, cells were treated with a PD 98059 compound at various time intervals (Fig. 6A), then treated with thrombin for 6 h. Inhibition of ERK activity using the PD compound also inhibited NF-B transactivation, suggesting a role of ERK phosphorylation in thrombin-induced NF-B activation (Fig. 6B). These results suggest an apparent association between thrombin-induced MIF induction, ERK phosphorylation and NF-B dependent transactivation.

DISCUSSION

Although early signaling events that occur following thrombin receptor binding are well described [Coughlin, 1999; Kataoka et al., 2003], the molecular events that ultimately result in thrombin-induced EC activation and nuclear signaling are not completely understood. Increasing information indicates that cytoskeletal proteins and other intracellular events that affect cytoskeletal organization are important targets for thrombin-activated signaling pathways [Borbiev et al., 2003; Wadgaonkar et al., 2003]. Our results have shown that thrombin induces the secretion of MIF, which is required for biphasic ERK activation. Myosin light chain kinase (MLCK)-driven cytoskeletal events appear to be critical in MIF secretion, as the MLCK inhibitor (ML7) attenuates MIF secretion. However, the precise mechanisms by which MIF is secreted and upregulated in the endothelial cells is unknown. MIF-induced ERK phosphorylation, at least in the initial stages of recombinant human MIF interaction with the endothelium, is inhibited by ML7, and may involve MLCK activation. In the later stages however, sustained ERK phosphorylation is not inhibited by ML7, and may have a different mechanism for activation. This suggests that MLCK activation may

be primarily involved in MIF secretion. These results are of particular interest, as we have previously shown the direct functional interaction of MIF with endothelial MLCK [Wadgaonkar et al., 2005a]. MIF binding to MLCK does not appear to increase EC MLCK enzymatic activity, suggesting an alternate, novel role for this interaction. One possibility is that MIF directly protects MLCK from apoptotic cleavage induced by caspases, an event which we have demonstrated to result in the generation of a constitutively active MLCK fragment, without regulation by Ca^{++} availability [Wadgaonkar et al., 2005b]. Further studies are needed to examine the mechanism of MIF- dependent protection of EC's from apoptosis, as well as the mechanism by which MIF activates NF-B transactivation.

Several studies have shown a role for extracellular MIF in protecting cells from apoptosis, including EC protection from the death-inducing effects of lipopolysaccharide (LPS), possibly via regulation of key apoptotic effectors [Calandra et al., 2000; Chagnon et al., 2005]. However, the pathways regulating MIF secretion are not well understood. Our present preliminary data suggests a role for MLCK in controlling the release of intracellular MIF from activated endothelial cells. The precise role of secreted MIF in EC protection from apoptosis is unknown, and is the subject of our ongoing research. The signaling molecules which control the balance between cell survival and apoptotic pathways are also not well understood, but there is increased appreciation that MLCK activation-dependent actin cytoskeleton changes may participate in determining the cellular fate of the thrombin-challenged endothelium.

Since the MIF gene does not encode an N-terminal signal sequence for translocation to the endoplasmic reticulum (ER) or golgi network, it is not clear how MIF secretion is regulated. Several studies have speculated non-classical pathways for MIF secretion, including ABCA1 dependent transport [Eickhoff et al., 2001; Flieger et al., 2003]. We have noted that, within 15 min of thrombin treatment in ECs, MIF colocalized with myosin light chain and actin stress fibers [Wadgaonkar et al., 2005a]. Also, some membrane localization was noted. After 30 min major staining was noted on cell surface [Wadgaonkar et al., unpublished data]. Major thrust of this paper is to show the correlation between myosin light chain kinase (MLCK) activation and MIF secretion. Thrombin is known to activate MLCK and use ABCA1 for endothelial cell dependent platelet activation. These supportive studies may help to correlate thrombin dependent MIF secretion with ABC transporters. However further studies will be required to identify the exact pathway for thrombin dependent MIF secretion. Recent studies have identified a novel role for MIF in the regulation of leukocyte recruitment to endothelial cells during atherosclerosis [Bernhagen et al., 2007]. These studies have characterized MIF as a functional noncognate ligand for the chemokine receptors CXCR2 and CXCR4, thereby regulating inflammatory and atherogenic leukocyte recruitment [Bernhagen et al., 2007]. However, it is not clear if MAP kinase activation induced by MIF is CXCR or CD44-CD74 dependent. Interestingly, CXCR and CD74 receptors are present on pulmonary endothelial cells. In our future studies, we hope to characterize the role of CXCR receptors in thrombin dependent MIF signaling.

Extracellular regulated kinase (ERK), a mitogen-activated protein kinase (MAPK), is regulated by threonine and tyrosine, with ERK phosphorylation activating nuclear transcription factors which control immediate early gene expression. In addition, activation of ERK is correlated with direct phosphorylation of MLCK, a process involved in EC migration [Borbiev et al., 2003; Wadgaonkar et al., 2005b], indicating that MLCK may be located either upstream or downstream from ERK [Borbiev et al., 2003]. In the present study, we report that thrombin evokes a biphasic ERK activation in a time-dependent manner, as demonstrated by biochemical analysis. The biphasic ERK½ phosphorylation is regulated by a PKC-dependent mechanism as well as MIF dependent signaling. Activation of these MAPK family members depends on the phosphorylation of threonine and tyrosine residues in a TXY motif highly specific for the ERK½ dual specificity MAPK kinase (MEK1/2), although other pathways of ERK activation may also occur [Seger and Krebs, 1995]. Although thrombin- induced biphasic ERK½ activation in endothelial cells is a novel pathway, similar biphasic activation has been reported for other stimuli like H_2O_2, basic fibroblast growth factor, and nerve growth factor [Meloche et al., 1992; York et al., 1998; Fukuzawa et al., 2002]. Earlier reports have shown that the Ras/Raf/MEK cascade is known as an ERK½ activation system, and that a specific MEK inhibitor can abolish thrombin-induced ERK activation, suggesting the predominant pathway of ERK activation after treatment with thrombin is via phosphorylation by MEK [Seger and Krebs 1995; Borbiev et al., 2003]. Inhibition of ERK activation by its specific inhibitor PD98059, in endothelial cells has shown to regulate thrombin-induced expression of PDGF-B, VCAM-1 and E-selectin gene expression, suggesting that ERK activity plays a significant role in the thrombin-signaling pathway in endothelial cell-gene activation. Therefore, MIF-dependent activation of ERK represents an important link between thrombin dependent ERK phosphorylation and transcriptional regulation, most likely by NF-B as we have shown.

Several reports show the involvement of MIF in NF-B dependent transactivation [Duan and Cannon, 2000; Lacey et al., 2003; Amin et al., 2006]. Duan and Cannon [2000] have reported that MIF antagonizes the anti-inflammatory effects of hydrocortisone in mononuclear cells by increasing NF-B activation [Duan and Cannon, 2001]. However, Kleemann et al. [2000] have not noticed any difference in TNF induced NF-B activation after MIF treatment. Lacey et al. [2003] have reported that MIF regulates fibroblast proliferation via ERK1/2 but not via NF-B activation [Lacey et al., 2003]. MIF activates VCAM-1 and ICAM-1 expression via the NFkB pathway which up-regulates several proinflammatory genes. Our results support this role of MIF secretion and ERK-dependent NFkB activation in a BPAE cell line. We also observed MIF dependent ERK phosphorylation in human pulmonary artery endothelial cells, suggesting a more common role of MIF secretion in cellular signaling.

In summary, we have demonstrated that thrombin activates ERK½ in a biphasic manner in endothelial cells. Secretion of MIF from endothelial cells contributes to the late phase activation of ERK½ by thrombin. Our present hypothesis, as shown in Figure 7 is that thrombin induced MIF secretion initiates its binding to the endothelial cell MIF receptor. This triggers the sustained activation

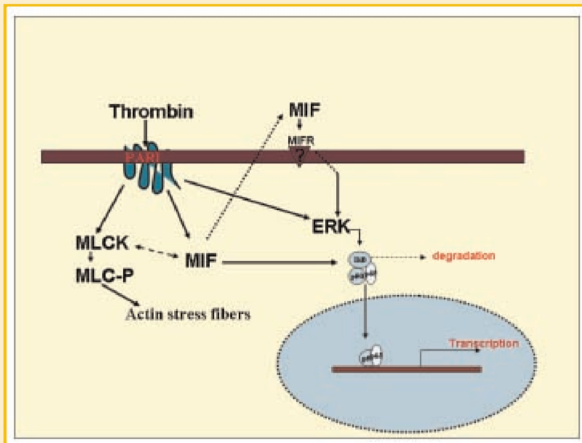

Fig. 7. Intracellular pathways regulated by MIF–MLCK interaction. Thrombin induced endothelial MIF interacts with cytoskeleton associated myosin light chain kinase (MLCK) and trigger ERK1/2 phosphorylation dependent nuclear signaling. Thrombin induced MIF secretion initiates its binding to the endothelial receptor. This also triggers the sustained activation of ERK½, which further generates NF-B dependent transcriptional regulation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of ERK½, which further generates a NF-B dependent transcriptional regulation. In conclusion, thrombin, an important mediator of vascular diseases, can also activate several pathways, which require NF-B dependent transcription, leading to the synthesis of multiple adhesion molecules and subsequent recruitment of multiple cell types.

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