Dynasore, a Dynamin Inhibitor, Induces PAI-1 Expression in MeT-5A Human Pleural Mesothelial Cells

Chi-Li Chung[1]*, Joen-Rong Sheu[2]*, Hsiang-Erh Liu[2], Shi-Chuan Chang[3], Yung-Chen Chou[2], Wei-Lin Chen[3], Duen-Suey Chou[2], and George Hsiao[2]

[1]Department of Chest Medicine, Taipei Medical University Hospital and School of Respiratory Therapy, College of Medicine, Taipei Medical University, Taipei, Taiwan; [2]Graduate Institute of Medical Sciences and Department of Pharmacology, College of Medicine, Taipei Medical University, Taipei, Taiwan; and [3]Institute of Emergency and Critical Care Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan

Plasminogen activator inhibitor-1 (PAI-1) is a primary regulator of plasminogen activation that plays an essential role in regulating the physiological thrombotic/fibrinogenic balance. The elevation of PAI-1 expression by human pleural mesothelial cells has been reported to contribute to pleural fibrosis and pleurodesis. In this study, we examined the effects on PAI-1 expression of dynasore, a cell-permeable inhibitor of dynamin, and its mechanisms in a human pleural mesothelial cell line (MeT-5A). The results indicated that dynasore enhanced transforming growth factor (TGF)-β_1– and TNF-α–induced PAI-1 protein expression in a concentration-dependent manner. Furthermore, dynasore significantly up-regulated PAI-1 protein and its messenger RNA expressions. Interestingly, Smad2/3 activation was induced by TGF-β_1 but not by dynasore. Among signaling inhibitors, a c-Jun NH_2-terminal kinase (JNK) inhibitor (SP600125) markedly attenuated dynasore-stimulated PAI-1 protein production. Consistently, dynasore strongly increased JNK phosphorylation. On the other hand, there was no enhancement effect by dynasore on TGF-β_1–induced matrix metalloproteinase-2 activation. These findings suggest that dynasore may stimulate PAI-1 protein expression and enhance TGF-β_1 activity through activation of JNK-mediated signaling in human pleural mesothelial cells. Given the profibrotic effect of dynasore, further *in vivo* studies may be conducted to evaluate its potential as a pleurodesing agent.

Keywords: dynasore; PAI-1; TGF-β1; JNK; MMP-2

Transforming growth factor-β_1 (TGF-β_1), a potent fibrogenic cytokine, has been reported to play a pivotal role in the development of pleural fibrosis, and is a crucial mediator of successful pleurodesis (1, 2). When stimulated *in vitro* by TGF-β, human pleural mesothelial cells secrete plasminogen activator inhibitor type 1 (PAI-1) (3). TGF-β signaling for PAI-1 production through the Smad pathway has been well described in a variety of cells (4). However, TGF-β signaling pathway for PAI-1 expression remains unclear in human pleural mesothelial cells.

Recently, there is increasing evidence that not only the Smad pathway but also others such as mitogen-activated protein kinase (MAPK) pathways are important in TGF-β signaling (5, 6). TGF-β superfamily members signal through heteromeric complexes of type I and II transmembrane Ser-Thr kinase receptors, which can be internalized either by a clathrin- or caveolae-dependent pathway (7). Depending on the entry route, the fates of internalized TGF-β receptors differ. Internalization via the clathrin pathway triggers signaling from early endosomes to express TGF-β–dependent proteins, whereas lipid raft/caveolar internalization may mediate the ubiquitin-dependent degradation of TGF-β receptors (7, 8).

Dynasore, a newly discovered cell-permeable dynamin inhibitor, rapidly blocks the formation of clathrin-coated vesicles (CCVs) through its inhibitory effects on the GTPase activity of dynamin (9). Dynasore noncompetitively inhibits the basal and stimulates rates of GTP hydrolysis without dramatically changing the affinity for GTP binding. This blockade is reversible and specific for dynamin-dependent endocytosis at plasma membranes. Since recent studies have indicated that endocytic organelles play a direct role in signal propagation and amplification (10), we hypothesized that dynasore, through its inhibition of dynamin-mediated endocytosis, may affect TGF-β–dependent cellular signaling pathways and the subsequent PAI-1 expression.

To the best of our knowledge, the effect of a dynamin inhibitor of TGF-β signaling on PAI-1 expression has never been investigated. The aim of this study was to verify the effects of dynasore on TGF-β activity and PAI-1 expression in MeT-5A human pleural mesothelial cells and its mechanisms.

CLINICAL RELEVANCE

Dynasore, as a dynamin inhibitor, stimulates and enhances transforming growth factor-β_1–induced plasminogen activator inhibitor-1 expression, and its main mechanism might be through c-Jun NH_2-terminal kinase signal pathway in human MeT-5A cells. It is proposed that dynasore may be used as a potential pleurodensing agent.

MATERIALS AND METHODS

Materials

N′-(3,4-Dihydroxybenzylidene)-3-hydroxy-2-naphthahydrazide, also called dynasore, a cell-permeable inhibitor of dynamin, was purchased from ChemBridge (San Diego, CA) and dissolved in dimethyl sulfoxide (DMSO). In each experiment, DMSO was employed at a constant final concentration of 0.1% or 0.2% (vol/vol). Chlorpromazine, thiazolyl blue tetrazolium bromide (MTT), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), sodium dodecylsulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, leupeptin, aprotinin, sodium fluoride, sodium orthovanadate, sodium pyrophosphate, diethyl pyrocarbonate (DEPC), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human TGF-β_1 and TNF-α were from Pepro Tech EC (London, UK). SB203580, SP600125, PD98059, and LY294002 were obtained from Calbiochem (San Diego, CA). All other chemicals used in this study were of reagent grade.

Cell Culture

MeT-5A cells (#CRL-9444TM), a human pleural mesothelial cell line, were obtained from American Type Culture Collection (ATCC, Man-

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Correspondence and requests for reprints should be addressed to George Hsiao, Ph.D., Graduate Institute of Medical Sciences and Department of Pharmacology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan. E-mail: geohsiao@tmu.edu.tw

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assas, VA) and grown in medium 199 (GIBCO, Invitrogen, San Diego, CA), supplemented with 20 mM HEPES, 24 mM sodium bicarbonate, 3.3 nM epidermal growth factor (EGF), 100 nM hydrocortisone, 4 mg/l insulin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere with 5% CO_2 incubator at 37°C. For subculturing, confluent dishes were detached by TrypLE Express (without phenol red; GIBCO), then neutralized with complete medium, at a split ratio of 1:3 in 100- × 20-mm flat-bottomed tissue Petri dishes (Orange Scientific, Braine-l'Alleud, Belgium) every 3 days. Throughout the experiments, cells were used between passages 18 and 23 as they originated from ATCC. Before the experiments, trypsinized MeT-5A cells were seeded at a density of 2.4×10^4 cells/cm^2 in the 60- × 15-mm flat-bottomed tissue Petri dishes. After the 2 days required to reach 70 to 80% confluence, cells were changed to serum-deprived medium for 24 hours, then subjected to the indicated treatments.

Cellular MTT Reduction

Cellular viability of MeT-5A cells after 24 hours of continuous exposure to dynasore (1–20 μM) was measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT as described previously (11). The percent cell viability was calculated as the absorbance of treated cells/control cells × 100%.

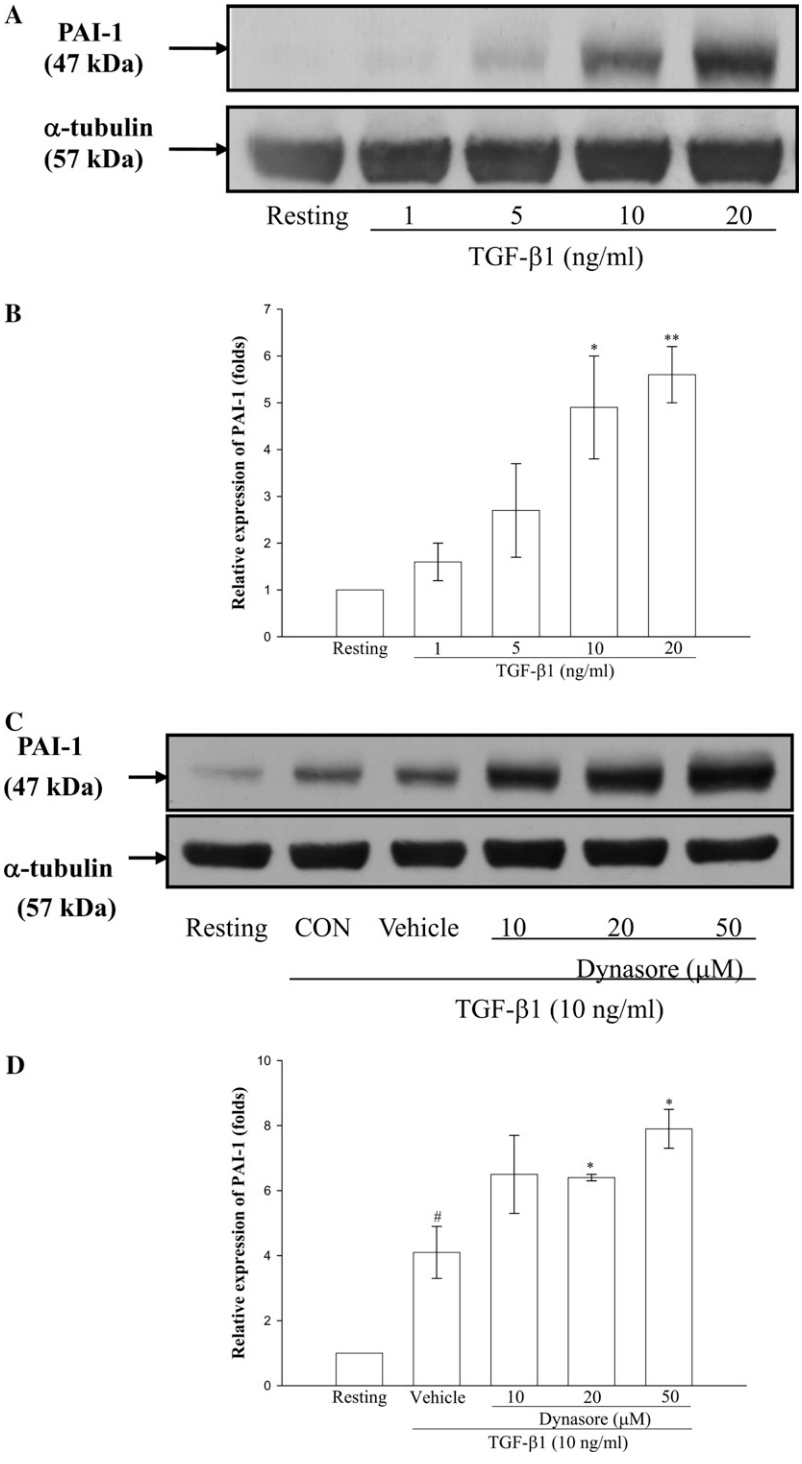

Figure 1. Enhancing effect of dynasore on transforming growth factor (TGF)-β_1–induced plasminogen activator inhibitor (PAI)-1 expression in MeT-5A cells. (*A*) MeT-5A cells (5×10^5 cells) were grown to 70 to 80% confluence and changed to serum-deprived medium for 24 hours, then stimulated with the indicated concentrations of TGF-β_1 for 24 hours. Total proteins from cell lysates were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and developed by Western blotting. Western blot analyses were performed using anti–PAI-1 and anti–α-tubulin antibodies. (*B*) Densitometric data are shown as the mean ± SEM of three independent experiments. *$P < 0.05$ and **$P < 0.01$, compared with the resting group. (*C*) MeT-5A cells were pretreated with the vehicle (DMSO 0.1%, vol/vol) or dynasore (10, 20, and 50 μM), then stimulated by TGF-β_1 (10 ng/ml) for 24 hours. Western blot analyses were performed using the same antibodies. (*D*) Data are shown as the mean ± SEM of three independent experiments. CON, the control group without vehicle treatment. $^{\#}P < 0.05$, compared with the resting group; *$P < 0.05$, compared with the vehicle group.

Western Blot Analysis

To determine the expressions of PAI-1, c-Jun NH_2-terminal kinase (JNK), and Smads in MeT-5A cells, Western blot analyses were performed as previously described (12). Briefly, MeT-5A cells were cultured in 60- × 15-mm Petri dishes. After reaching 70 to 80% confluence, culture dishes were changed to serum-deprived medium for 24 hours. Next, cells were treated with the vehicle (DMSO), indicated concentrations of dynasore, MAPK, or phosphatidylinositol 3-kinase (PI3K) inhibitors, and/or TGF-β_1 for the indicated times. In some experiments, anti–TGF-β mAb (monoclonal antibody) (1D11; R&D Systems, Minneapolis, MN) was added to the culture medium before treatment of dynasore. After incubation, cells were washed with ice-cold phosphate-buffered saline (PBS, pH 7.3). Proteins were extracted with lysis buffer (10 mM Tris-HCl, 140 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, 2 mM PMSF, 1 mM aprotinin, and 1 mM leupeptin; pH 7.0) for 30 minutes. In addition, phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, and 5 mM sodium pyrophosphate) were added to the lysis buffer for the phosphorylated JNK or Smad analysis. Lysates were centrifuged, and the supernatant (80 μg protein for PAI-1 and 50 μg protein for JNK or Smad) was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred onto nitrocellulose membranes (for PAI-1 proteins) or polyvinylidene difluoride (PVDF) membranes (for JNK or Smad proteins). After incubation in blocking buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20, and 5% dry skim milk; pH 7.5) overnight at 4°C and being washed three times with TBST buffer (10 mM Tris-base, 100 mM NaCl, and 0.1% Tween 20; pH 7.5), blots were treated with either an anti–PAI-1 mAb (1:2,000; BD Biosciences, San Jose, CA), anti-JNK mAb (1:2,000; Cell Signaling Technology, Beverly, MA), or an anti-Smad mAb (1:3,000; Cell Signaling Technology) in TBST buffer for 3 hours. They were subsequently washed three times with TBST buffer and incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit Abs (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 hours. Blots were then washed three times, and the band with peroxidase activity was detected using film exposure with enhanced chemiluminescence detection reagents (ECL^+ system; Amersham). The Digital Scanning System (PowerLook III/MagicScan V4.5; UMAX Data System, Taipei, Taiwan) with BIO-PROFIL Bio-1D light analytical software (Vilber Lourmat, Marue La Vallee, France) was used for the quantitative densitometric analysis. Data of specific protein levels were presented as relative multiples in relation to the control.

Isolation of Total RNA and Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from MeT-5A cells by a commercially available kit according to the manufacturer's instructions (TRIzol reagent; GIBCO). For each RT-PCR reaction, 0.8 μg of the RNA sample and 0.2 μM of primers were reverse-transcribed and amplified in a 50-μl reaction mixture of commercially available reagents (Super Script On-Step RT-PCR

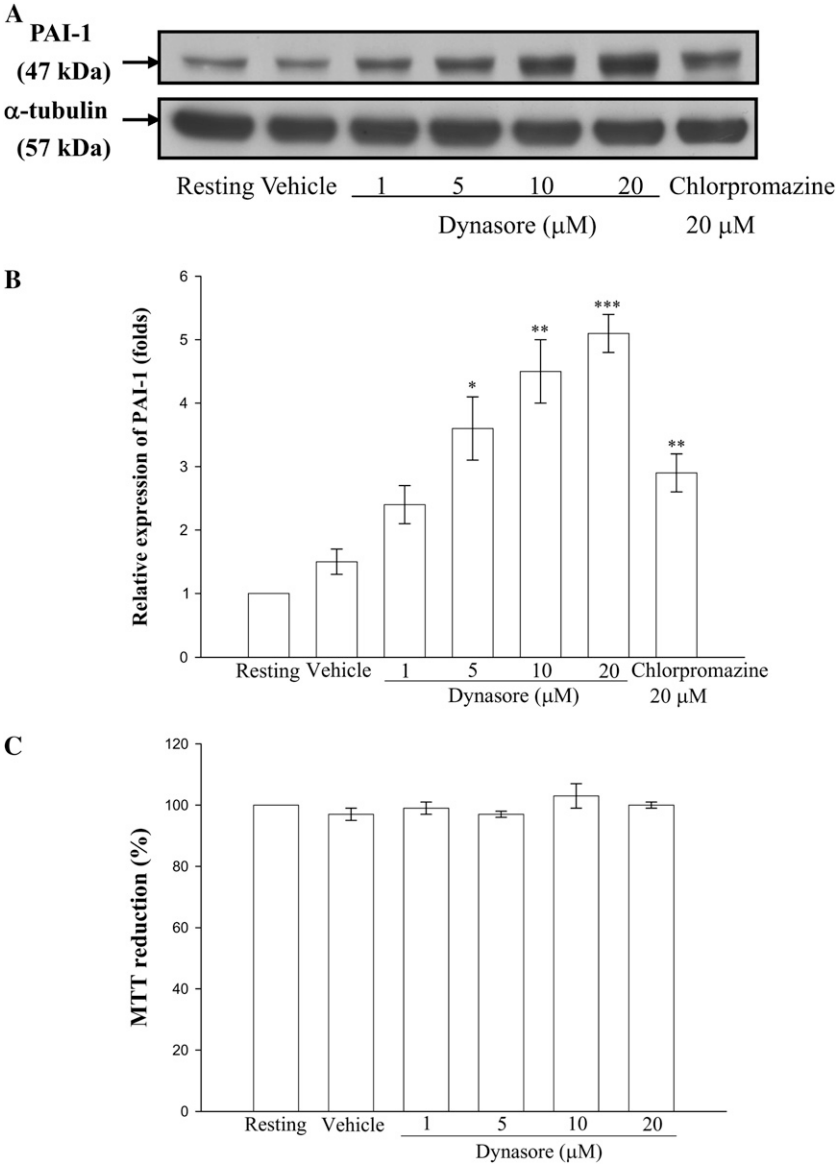

Figure 2. Effect of dynasore on PAI-1 expression and MTT reduction in MeT-5A cells. (*A*) MeT-5A cells (5×10^5 cells) were grown to 70 to 80% confluence and changed to serum-deprived medium for 24 hours, then treated in the absence or presence of vehicle (DMSO 0.1%, vol/vol), dynasore (1–20 μM), or chlorpromazine (20 μM) for 24 hours. Western blot analyses were performed using anti–PAI-1 and anti–α-tubulin antibodies. (*B*) Densitometric data are shown as the mean ± SEM of three to five independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, compared with the vehicle group. (*C*) Percentage of cellular MTT reduction is presented as the mean ± SEM of three independent experiments.

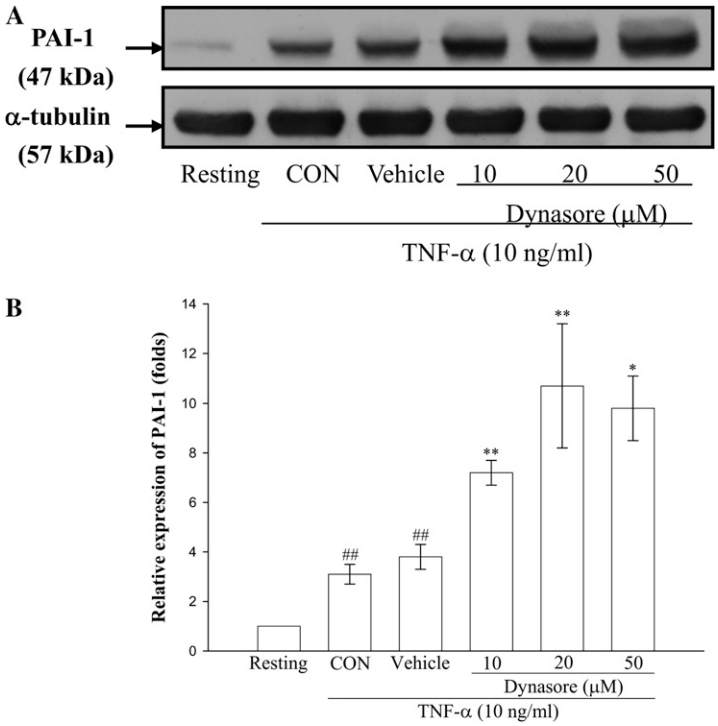

Figure 3. Enhancing effect of dynasore on TNF-α–induced PAI-1 expression in MeT-5A cells. (*A*) MeT-5A cells were pretreated with the vehicle (DMSO 0.1%, vol/vol) or dynasore (10, 20, and 50 μM), then stimulated by TNF-α (10 ng/ml) for 24 hours. Western blot analyses were performed using anti–PAI-1 and anti–α-tubulin antibodies. (*B*) Data are shown as the mean ± SEM of three independent experiments. CON, the control group without vehicle treatment. $^{\#\#}P < 0.01$, compared with the resting group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the vehicle group.

system; GIBCO). For PAI-1 cDNA production and amplification, reaction mixtures were subjected to 30 minutes at 50°C and 2 minutes at 94°C for reverse-transcription processes, followed by 25 cycles of 95, 60, and 72°C for 15, 30, and 30 seconds, respectively, and a final extension step at 72°C for 5 minutes in a Perkin-Elmer 2400 thermal cycler (Applied Biosystems, Waltham, CA). The primers used to target the PAI-1 mRNA were 5′-TGCTGGTGAATGCCCTCTACT-3′ (sense) and 5′-CGGTCATT CCCAGGTTCTCTA-3′ (antisense). The GAPDH primers sets were 5′-GCCGCCTGGTCACCAGGGCTG-3′ (sense) and 5′-ATGGACTG TGGTCATGAGCCC-3′ (antisense). For visualization and quantification by densitometry of each RT-PCR reaction, a 10-μl aliquot with sample loading dye (25% glycerol, 0.25% bromophenol blue) was electrophoresed in a 1.5% agarose gel using a mini horizontal submarine unit (HE 33; Amersham) containing 0.5 μg/ml ethidium bromide to allow ultraviolet-induced fluorescence (TCP-20.M; Vilber Lourmat). Preliminary experiments were performed to determine the range of amplification cycles and beginning RNA substrate within the linear phase of the exponential increase of PCR products for each particular primer pair. The Photo-Print Digital Imaging System (IP-008-SP; Vilber Lourmat) with analytical software (BIO-PROFIL Bio-1D light) was used for the quantitative densitometric analysis of gel bands as described by Hsiao and coworkers (12). The specific bands were quantified according to their relative multiples of intensity.

Inducible Matrix Metalloproteinase-2 Gelatinolysis

At the end of the 24 hours of incubation, the conditioned media were collected and matrix metalloproteinase (MMP) gelatinolytic capacity

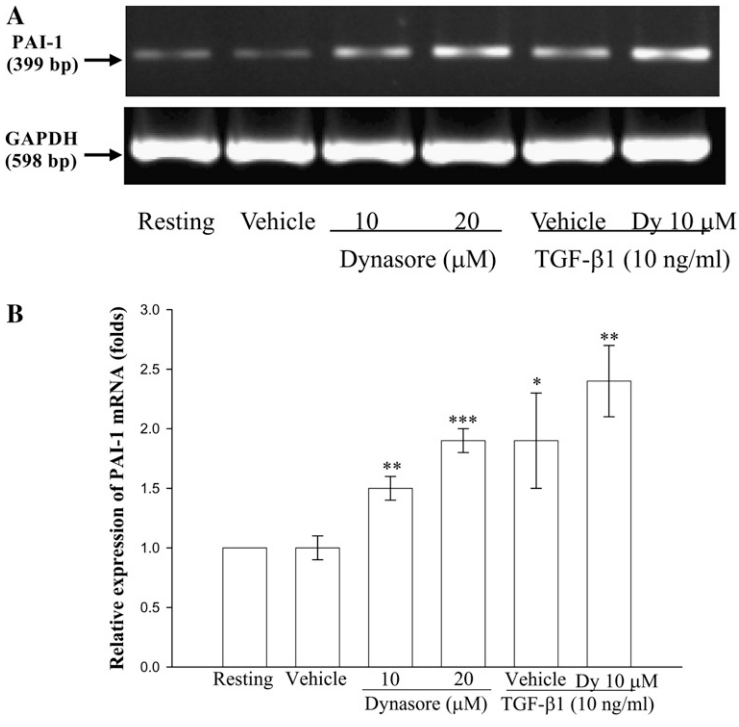

Figure 4. Effect of dynasore on PAI-1 mRNA expression in MeT-5A cells. (*A*) MeT-5A cells were treated with vehicle (DMSO 0.1%, vol/vol) and the indicated concentrations of dynasore (Dy, 10 or 20 μM), or stimulated with TGF-β_1 (10 ng/ml) for 6 hours. Total cellular RNA was isolated and applied to RT-PCR as described in Materials and Methods. PAI-1 mRNA levels were normalized with the endogenous control, GAPDH mRNA. (*B*) The relative multiples of densitometric data are shown as the mean ± SEM of three to six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the vehicle group.

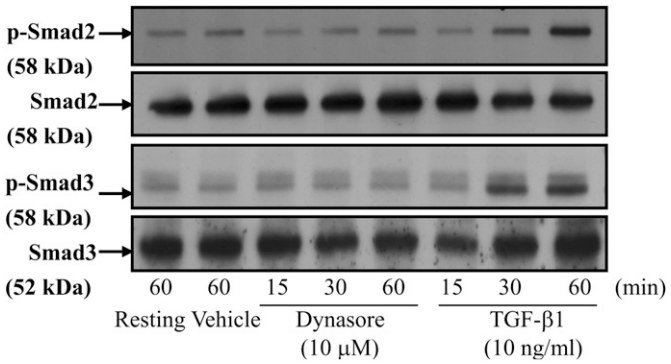

Figure 5. Effect of dynasore on Smad2/3 activation in MeT-5A cells. MeT-5A cells were treated with the vehicle or dynasore (10 μM) for 15, 30, and 60 minutes, then Smad2/3 activation was analyzed. TGF-β_1 (10 ng/ml) was added as the positive control for Smad activation. For the analysis of Smad2/3 activation, cells were lysed and immunoblotted with antibodies specific for either phosphorylated (p-Smad2/3) or total proteins. Results are representative examples of three independent experiments.

was evaluated as described by Zhang and colleagues (13) with some modifications. Briefly, the sample aliquot underwent electrophoresis on an SDS-polyarylamide (10%) gel with gelatin (1%) under nonreducing conditions. After three washes with 2.5% Triton X-100 buffer, the gels were incubated in reacting buffer (50 mM Tris-base, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij 35; pH 7.5) at 37°C for 48 hours. At the end of the reacting period, gels were incubated in a fixing solution (7% acetic acid and 40% methanol) and then stained with a solution of Coomassie brilliant blue G-colloidal. The specific clear bands, which were gelatinolytic sites, were analyzed using the same digital imaging system and analytical software as previously described. The appearance of gelatinolytic bands was completely inhibited by 10 mM EDTA (data not shown). HT-1080 (a human fibrosarcoma cell line) medium was used as the positive gelatinolytic control (14).

Statistical Analyses

The experimental results are expressed as the mean ± SEM and are accompanied by the number of observations. Data were assessed by Student's unpaired *t* test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Effect of Dynasore on TGF-β_1-Induced Expression of PAI-1 Protein in MeT-5A Cells

The immunoblotting analysis shown in Figure 1A revealed that TGF-β_1 (1–20 ng/ml) exerted a concentration-dependent stimulation of PAI-1 protein synthesis in human MeT-5A cells. According to these results, 10 ng/ml of TGF-β_1 was selected as a suitable concentration for further experiments (Figure 1B). Pretreatment with various concentrations of dynasore for 30 minutes before TGF-β_1 revealed that dynasore (10, 20, and 50 μM) concentration-dependently enhanced the production of PAI-1 protein stimulated by TGF-β_1 in MeT-5A cells (Figure 1C). As shown in Figure 1D, TGF-β_1–induced PAI-1 expression was markedly enhanced by dynasore (50 μM) by 7.9 ± 0.6-fold compared with 4.1 ± 0.8-fold of the vehicle group ($n = 3$).

Effect of Dynasore on the Expression of PAI-1 Protein and MTT Reduction in MeT-5A Cells

To verify the enhancing activity of dynasore on PAI-1 expression in TGF-β_1–stimulated MeT-5A cells, we first treated cells with dynasore alone to evaluate its intact action. As shown in Figures 2A and 2B, dynasore itself (1, 5, 10, and 20 μM) had a stimulatory effect on PAI-1 protein expression in a concentration-dependent manner by 2.4 ± 0.3-, 3.6 ± 0.5-, 4.5 ± 0.5-, and 5.1 ± 0.3-fold compared with the resting condition ($n = 3$–5). Interestingly, the classical endocytic inhibitor, chlorpromazine, induced an increase in PAI-1 expression of approximately 2.9 ± 0.3-fold at a concentration of 20 μM.

To further demonstrate whether dynasore affects the viability or mitogenic effect of MeT-5A cells, cells were preincubated with various concentrations of dynasore for 24 hours. According to the MTT assay, we found that dynasore (1, 5, 10, and 20 μM) had no significant effect on cellular proliferation or viability of MeT-5A cells (Figure 2C).

Effect of Dynasore on TNF-α–Induced Expression of PAI-1 Protein in MeT-5A Cells

To ascertain the enhancing role of dynasore, we used TNF-α as a second inducer for PAI-1 expression. As shown in Figure 3A, TNF-α (10 ng/ml) exerted a significant stimulation of PAI-1 protein synthesis in human MeT-5A cells. Pretreatment with various concentrations of dynasore for 30 minutes before TNF-α revealed that dynasore (10, 20, and 50 μM) concentration-dependently enhanced the production of PAI-1 protein in MeT-5A cells (Figure 3A). As shown in Figure 3B, TNF-α–induced PAI-1 expression was markedly potentiated by dynasore (10, 20, and 50 μM) by 7.2 ± 0.5-, 10.7 ± 2.5-, and 9.8 ± 1.3-fold compared with 3.8 ± 0.5-fold of the vehicle group ($n = 3$), respectively.

Effect of Dynasore on the Expression of PAI-1 mRNA in MeT-5A Cells

As shown in Figure 4A, dynasore (10 and 20 μM) significantly increased the expression of PAI-1 mRNA by approximately 50% and 90%, respectively, compared with the vehicle condition (DMSO, 0.1% vol/vol). TGF-β_1 (10 ng/ml) also markedly stimulated an increase in PAI-1 mRNA in MeT-5A cells compared with the resting condition. Furthermore, pretreatment with dynasore (10 μM) for 30 minutes enhanced TGF-β_1–induced PAI-1 mRNA expression by up to 2.4 ± 0.3-fold ($n = 3$) compared with the resting condition in MeT-5A cells (Figure 4B). The expression of mRNA was induced to a greater extent by the combination of dynasore and TGF-β_1 than that by single treatment (Figure 4B). These results revealed that either dynasore or TGF-β_1 significantly induces PAI-1 protein synthesis through activation of gene expression in MeT-5A cells, and dynasore may have an additive effect on PAI-1 mRNA expression induced by TGF-β_1.

Effects of Dynasore on Smad2/3 Activation

To verify whether the induction of PAI-1 expression by dynasore is mediated through Smad signaling, the effect of dynasore on Smad2/3 phosphorylation was examined. Phosphorylation of Smad2/3 is an obligatory step for TGF-β signaling (15). As shown in Figure 5, phosphorylation of Smad2 and Smad3 was significantly and time-dependently (15–60 min; Figure 5, *lanes 6–8*) increased by stimulation with TGF-β_1 (10 ng/ml) in MeT-5A cells, compared with the vehicle and resting conditions (Figure 5, *lanes 2* and *1*), respectively. However, dynasore (10 μM) itself had no significant effect on increasing the phosphorylation of Smad2 or Smad3 in MeT-5A cells (Figure 5, *lanes 3–5*).

Effects of MAPK and PI3K Inhibitors on Dynasore-Induced Expression of PAI-1 Protein in MeT-5A Cells

To further investigate the stimulatory mechanism of dynasore on PAI-1 expression in MeT-5A cells, we detected several TGF-β–dependent Smad-independent signaling molecules, including PI3K and MAPKs, by using their specific pharmacologic

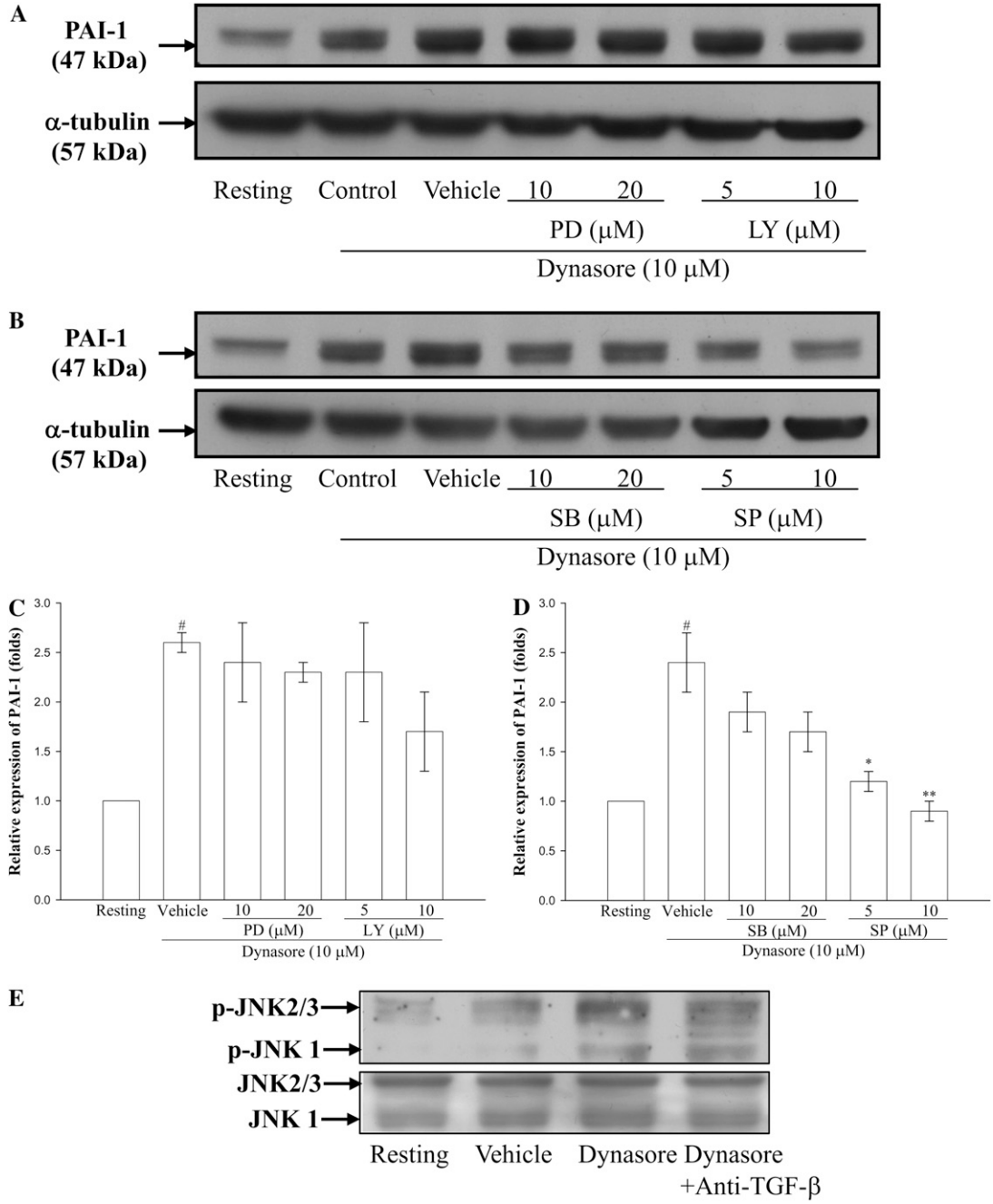

Figure 6. Effects of signaling inhibitors on dynasore-induced PAI-1 expression in MeT-5A cells. (*A* and *B*) MeT-5A cells were preincubated with the vehicle, PD98059 (PD, 10 and 20 μM), LY294002 (LY, 5 and 10 μM), SB203580 (SB, 10 and 20 μM), or SP600125 (SP, 5 and 10 μM), then dynasore (10 μM) was added for 24 hours. Western blot analyses were performed using anti-PAI-1 and anti-α-tubulin antibodies. (*C* and *D*) Relative multiples of densitometric data are expressed as the mean ± SEM of three independent experiments. $^{\#}P < 0.05$, compared with the resting group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the vehicle by dynasore (10 μM) stimulation. (*E*) After treatment with dynasore or the anti-human TGF-β antibody (30 μg/ml) with dynasore for 60 minutes, JNK activation was determined by Western blotting with either a monoclonal antibody which recognized phosphorylated or total JNK. Results are representative examples of five to six independent experiments.

inhibitors. According to the immunoblotting analysis shown in Figures 6A and 6B, neither pretreatment with an MEK inhibitor (PD98059; 10 and 20 μM), a PI3K inhibitor (LY294002; 5 and 10 μM), nor a p38 MAPK inhibitor (SB203580; 10 and 20 μM) markedly attenuated PAI-1 expression induced by dynasore (10 μM). In contrast, a JNK inhibitor (SP600125; 5 and 10 μM) significantly and concentration-dependently attenuated dynasore-stimulated PAI-1 protein production (Figure 6B). PAI-1 expression was mostly inhibited by SP600125 at a concentration of 10 μM (Figure 6D). Furthermore, as shown in Figure 6E, phosphorylation of JNK (2/3) was significantly increased by stimulation with dynasore (10 μM) by up to approximately 1.8-fold (n = 5–6) compared with the vehicle condition in MeT-5A cells. Moreover, this elevation of JNK (2/3) phosphorylation was strongly attenuated by the addition of an anti-human TGF-β mAb (30 μg/ml, 1D11) (Figure 6E).

Effects of Dynasore on TGF-β1–Induced MMP-2 Gelatinolysis in MeT-5A Cells

According to preliminary studies, TGF-β_1 (1, 5, 10, and 20 ng/ml) induced concentration-dependent increase in latent MMP-2 (92-kD)–mediated gelatinolysis in the culture medium of MeT-5A cells of 1.2 ± 0.1-, 1.9 ± 0.3-, 2.4 ± 0.3-, and 2.6 ± 0.2-fold compared with the resting condition, respectively (data not shown). After pretreatment of cells with various concentrations of dynasore (10, 20, and 50 μM) for 15 minutes followed by the addition of TGF-β_1 (10 ng/ml), we found that dynasore concentration-dependently inhibited MMP-2–mediated gelatinolysis stimulated by TGF-β_1 (Figure 7A). At 10, 20, and 50 μM, dynasore inhibited this gelatinolytic reaction by approximately 30.0 ± 3.8%, 48.6 ± 5.6%, and 87.7 ± 11.9%, respectively (Figure 7B).

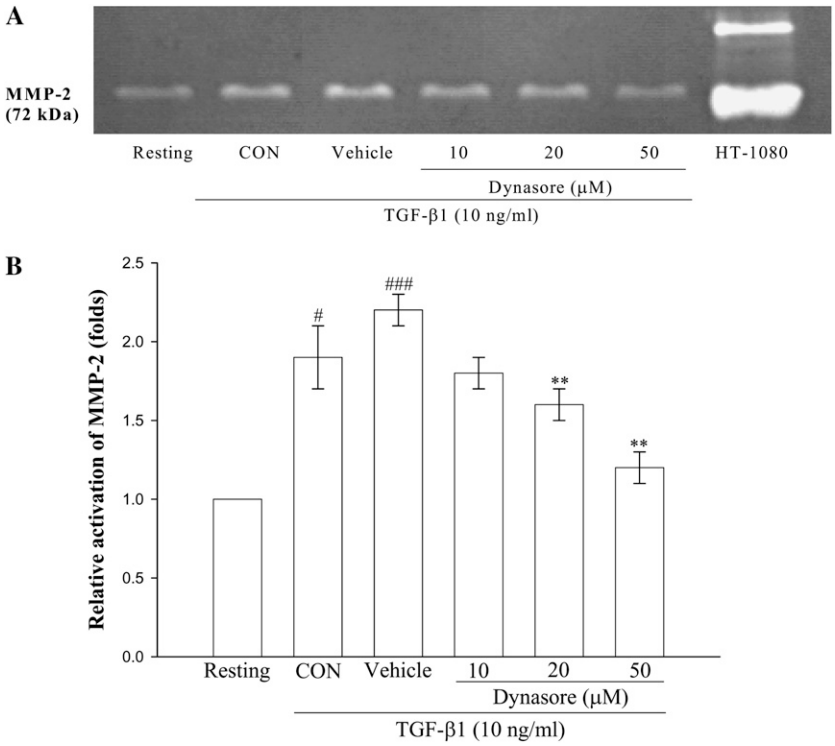

Figure 7. Effect of dynasore on TGF-β_1–induced MMP-2 activation in MeT-5A cells. (*A*) MeT-5A cells were treated with the vehicle or dynasore, and stimulated by TGF-β_1 (10 ng/ml) for 24 hours as indicated. Cells were treated with the indicated concentrations of dynasore (*lane 4*, 10 μM; *lane 5*, 20 μM; *lane 6*, 50 μM) or the vehicle (*lane 3*) for 15 minutes before treatment with TGF-β_1. Cell-free supernatants were then assayed for MMP-2 activity by gelatin zymography, as detailed in MATERIALS AND METHODS (*lane 2*, control). (*B*) The relative multiples of densitometric data are shown as the mean ± SEM of three independent experiments. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$, compared with the resting group; $^{**}P < 0.01$, compared with the vehicle group.

DISCUSSION

Much evidence has shown that PAI-1 is expressed in human pleural mesothelial cells in response to stimulation by proinflammatory cytokines or pleurodesing agents (16). Increased PAI-1 expression and decreased fibrinolysis have been found to be crucial for successful pleurodesis in malignant pleural effusions (17). Moreover, elevated PAI-1 expression is also implicated in the pathogenesis of pulmonary fibrosis (18). The present study demonstrates that dynasore may enhance PAI-1 expression induced by TGF-β_1 and TNF-α in MeT-5A human pleural mesothelial cells. In addition, dynasore itself may activate the JNK-mediated pathway, and increase PAI-1 mRNA and protein synthesis in MeT-5A cells. Furthermore, dynasore may inhibit the secretion of MMP-2 from MeT-5A cells stimulated by TGF-β_1.

The recent literature showed that TGF-β ligands induce receptor internalization in clathrin-coated endosomes, which depend on dynamin and may be required for efficient TGF-β signaling through Smads (19–21). Since dynasore was recently found to inhibit dynamin and the formation of endocytic clathrin-coated pits and vesicles (9, 22), we supposed that this compound can attenuate TGF-β_1 signaling and PAI-1 synthesis by inhibiting receptor internalization in MeT-5A cells. The results showed that PAI-1 expression was concentration-dependently induced by TGF-β_1 in MeT-5A cells in a manner similar to that in primary pleural mesothelial cells as demonstrated in a previous study (3). After the binding of TGF-β to the type II receptor, the type I receptor is phosphorylated, which further phosphorylates Smad2 and Smad3 to form heteromeric complexes with Smad4. These complexes translocate to the nucleus, where they regulate the expression of target genes (15). Interestingly, our findings revealed that dynasore did not attenuate TGF-β–activated PAI-1 production. On the contrary, TGF-β–stimulated production of PAI-1 protein and mRNA was enhanced by dynasore. Furthermore, dynasore itself exerted a stimulatory effect of increasing PAI-1 mRNA and protein production. It seems that several mechanisms may be operating in the enhancing effect of dynasore on the TGF-β/Smad signaling. It has been proposed that elevated Smad2/3 phosphorylation for enhancing TGF-β signaling results from either a reduction in the negative regulator, Smad7 (23), or a decrease in degradation of the phosphorylated Smad2/3 (24). However, our results showed that Smad2 and Smad3 phosphorylation was significantly induced by TGF-β but not by dynasore under the experimental conditions. In addition, recent research demonstrated that Smad2/3 is not involved in TGF-β_1 signaling in mesothelioma and mesothelial cells (25). It seems reasonable to assume that dynasore may have no direct action on Smad2/3 activation in MeT-5A cells. On the other hand, BMP and an activin membrane-bound inhibitor (Bambi) have been shown to act as an endogenous antagonist of TGF-β signaling (26). Moreover, recent reports demonstrated that the proinflammatory mediator can down-regulate Bambi production and thereby enhance TGF-β activation and fibrogenesis (27). Furthermore, a more complicated pathway of Smad1-dependent/Smad3-independent signaling on fibrogenic induction was recently described in fibroblasts (28). Determining whether dynasore exerts its action on Bambi inhibition or Smad1 activation for enhancement of TGF-β activation needs further investigation.

It is well known that dynamin is essential for both CCV and caveolar formation in endocytosis (29, 30). Therefore, altered TGF-β_1 function may be associated with trafficking of receptors between these two pools (31). According to a previous report (32), TGF-β activation is mediated by the association of the TGF-β receptor complex with clathrin-coated endosomes, leading to decreased association of the receptor with caveolar vesicles and reduced TGF-β receptor turnover. It may be that dynasore predominantly inhibits caveola-associated dynamin and results in internalization of TGF-β receptors to caveolar endosomes to a smaller degree than to CCVs, and thereby decreases turnover of the receptors and augments TGF-β signaling in MeT-5A cells. Further studies of TGF-β_1 receptor-associated endocytosis and receptor turnover due to stimulation by dynasore in MeT-5A cells needs to be verified.

However, besides Smad-mediated transcription, TGF-β activates other signaling cascades, including MAPK and PI3K pathways (33). TGF-β–induced activation of the ERK, JNK, and p38 pathways can result in Smad phosphorylation and regulate Smad activation (34–36). Furthermore, LY294002, an inhibitor of PI3K, blocks TGF-β_1–induced Smad2/3 phosphorylation, suggesting that activation of PI3K by TGF-β_1 may regulate Smad signaling (37, 38). We demonstrated in this study that LY294002 had little effect on dynasore-induced PAI-1 expression, which may imply that the signaling of dynasore activation is not similar to that of TGF-β on the Smad-dependent pathway. A recent study showed that TGF-β_1 is essential for mesothelioma and mesothelial cells to produce pro–MMP-2 via a p38 MAPK–dependent pathway (25). These results reveal the inhibitory effect of dynasore on MMP-2 secretion, which indicates that dynasore-induced MeT-5A cell activation might not be dependent on the p38 MAPK-mediated pathway.

It is well known that the PAI-1 promoter contains binding sites for Smads, c-Jun/c-Fos heterodimers (AP-1), and NF-κB (39, 40). JNK MAPK-activated transcription factors such as AP-1 interact with Smad complexes and facilitate DNA binding (41). It has also been shown that transcriptional activation by Smad is mediated through the AP-1 transcription factor complex (6). Furthermore, different activators, such as thrombin (42), angiotensin II (43), and oxidative stress (44), have been reported to increase PAI-1 expression through a Smad-independent signaling pathway of JNK activation. Especially, an *in vivo* study using the JNK inhibitor, SP10025, revealed that aerosolized lipopolysaccharide (LPS) increased pulmonary PAI-1 expression through a JNK-mediated pathway (45). Therefore, we investigated the role of Smad-independent pathways of dynasore-induced PAI-1 protein expression in MeT-5A cells. The results revealed that only SP600125 markedly attenuated dynasore-induced PAI-1 production. Consistently, dynasore significantly induced JNK phosphorylation in MeT-5A cells. Interestingly, this dynasore-induced JNK activation was attenuated by an anti–TGF-β antibody with binding inhibition. These findings suggest that a TGF-β–related, JNK-dependent pathway is involved in dynasore-induced PAI-1 production. Interestingly, it was also found that the classical endocytic inhibitor, chlorpromazine (46), could induce PAI-1 expression but less than that of dynasore at the same concentration in the experimental conditions. This implies that inhibition of endocytosis may also play a crucial role in regulating PAI-1 production. In this study, we also showed that TNF-α stimulates PAI-1 production in pleural mesothelial cells, similar to what was shown in a previous study by Idell and coworkers (3). TNF-α induces PAI-1 expression via activation of NF-κB and MAPK in vascular endothelial cells (47). It was clearly found that TNF-α–stimulated production of PAI-1 was also enhanced by dynasore. This strongly demonstrates that dynasore can at least activate the common pathway of PAI-1 expression shared by different stimulators. Moreover, there are also a variety of mechanisms other than TGF-β signaling that induce PAI-1 overexpression, such as specific tyrosine kinase– (48), Rho-kinase– (49), and JAK/STAT-dependent (50) pathways in different cells. Whether other transcription factors and signal transducers of transcription are involved in the dynasore-mediated pathway has yet to be elucidated.

Recently, it was shown that talc, the most commonly used pleurodesing agent in clinical practice, can increase TGF-β_1 production by pleural mesothelial cells (51). However, the induction of TGF-β_1 by dynasore in MeT-5A cells was not evaluated in this study. According to our results, Smad2/3 was activated by exogenous TGF-β_1, but not by dynasore during a treatment period of 60 minutes. On the other hand, TGF-β_1–induced MMP-2 activation was not enhanced by dynasore treatment for 24 hours in MeT-5A cells. These findings might not be sufficient to suggest that TGF-β_1 was not produced on stimulation by dynasore during short- or long-term treatment. Furthermore, direct intrapleural administration of TGF-β induces excellent pleurodesis in different animal models (52, 53), which suggests that TGF-β may be used clinically as a pleurodesing agent. The present study demonstrated that dynasore not only enhanced TGF-β_1–induced PAI-1 expression but also directly stimulated PAI-1 expression in MeT-5A cells. Further studies are warranted to clarify the role of dynasore in pleural fibrosis and its potential use as a pleurodesing agent.

In conclusion, the present study indicates that dynasore, a potent inhibitor of endocytic pathways known to depend on dynamin, may stimulate PAI-1 protein expression and enhance TGF-β1 activity through activation of JNK-mediated signaling in human pleural mesothelial cells. Given the profibrotic effect of dynasore, further studies should be conducted to evaluate its potential to be a pleurodesing agent *in vivo*.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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