Inhibitors of clathrin-dependent endocytosis enhance TGFβ signaling and responses

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Summary

Clathrin-dependent endocytosis is believed to be involved in TGFβ-stimulated cellular responses, but the subcellular locus at which TGFβ induces signaling remains unclear. Here, we demonstrate that inhibitors of clathrin-dependent endocytosis, which are known to arrest the progression of endocytosis at coated-pit stages, inhibit internalization of cell-surface-bound TGFβ and promote colocalization and accumulation of TβR-I and SARA at the plasma membrane. These inhibitors enhance TGFβ-induced signaling and cellular responses (Smad2 phosphorylation/nuclear localization and expression of PAI-1). Dynasore, a newly identified inhibitor of dynamin GTPase activity, is one of the most potent inhibitors among those tested and, furthermore, is a potent enhancer of TGFβ. Dynasore ameliorates atherosclerosis in the aortic endothelium of hypercholesterolemic *ApoE*-null mice by counteracting the suppressed TGFβ responsiveness caused by the hypercholesterolemia, presumably acting through its effect on TGFβ endocytosis and signaling in vascular cells.

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Key words: Endocytosis inhibitor, TGFβ, Coated pit, Signaling, Enhancer, Atherosclerosis

Introduction

Transforming growth factor beta (TGFβ) comprises a family of pleiotropic cytokines, which includes TGFβ1, -β2 and -β3 in mammals, that function as bifunctional growth regulators (Roberts, 1998). They inhibit the growth of most cell types, including epithelial cells, endothelial cells and lymphocytes but stimulate the growth of mesenchymal cells. The growth-regulatory activity of TGFβ has been implicated in carcinogenesis, immunomodulation and cellular differentiation. TGFβ is the most potent known stimulator of synthesis and deposition of extracellular matrix and plays an important role in wound healing and tissue fibrosis. It has anti-inflammatory and pro-inflammatory activities, depending on the tissue studied. Because of its anti-inflammatory and immunomodulatory activities, TGFβ in blood is a protective cytokine for atherosclerosis in the cardiovascular system (Chen et al., 2007; Metcalfe and Grainger, 1995).

TGFβ stimulates cellular responses by inducing formation of a hetero-oligomeric TGFβ receptor complex at the plasma membrane (Heldin et al., 1997; Massague, 1998). Within this complex, the constitutively active type II TGFβ receptor (TβR-II) phosphorylates and activates the type I TGFβ receptor (TβR-I). The activated TβR-I phosphorylates Smad2 and Smad3; the phosphorylation is facilitated by the Smad anchor protein called 'Smad anchor for receptor activation' (SARA) (Tsukazaki et al., 1998; Xu et al., 2000). Phosphorylated Smad2–Smad3 associates with Smad4 to form heterotrimeric complexes that translocate to and accumulate in the nucleus, where they regulate transcription of responsive genes. Smad7, a negative regulator of TGFβ signaling, is associated with lipid rafts/caveolae and mediates degradation of TGFβ bound to the TGFβ receptor (Di Guglielmo et al., 2003; Ito et al., 2004). The cellular responses to TGFβ are determined by TGFβ partitioning between clathrin-dependent and caveolae-dependent endocytosis pathways (Chen et al., 2006; Chen et al., 2007; Chen et al., 2008; Di Guglielmo et al., 2003; Huang and Huang, 2005; Ito et al., 2004; Le Roy and Wrana, 2005). The former promotes signaling and cellular responses, whereas the latter leads to rapid degradation of TGFβ-bound TGFβ receptors and attenuation of TGFβ responsiveness (Chen et al., 2006; Chen et al., 2007; Chen et al., 2008; Di Guglielmo et al., 2003; Huang and Huang, 2005; Ito et al., 2004; Le Roy and Wrana, 2005). Although clathrin-dependent endocytosis is involved in signaling (Chen et al., 2007; Hayes et al., 2002; Huang and Huang, 2005; Mitchell et al., 2004; Penheiter et al., 2002), the subcellular locus where TGFβ induces signaling remains unclear (Lu et al., 2002).

Endosomes are believed to be important mediators of TGFβ-induced signaling (Chen et al., 2006; Chen et al., 2007; Di Guglielmo et al., 2003; Ito et al., 2004). This is based on the observations that TGFβ receptor internalization and TGFβ-induced cellular responses are inhibited by overexpression of dynamin dominant-negative mutant K44A (Di Guglielmo et al., 2003) and that SARA colocalizes with endosome markers in endosomes (Di Guglielmo et al., 2003; Hayes et al., 2002). However, Lu et al. (Lu et al., 2002) demonstrated that overexpression of dynamin K44A inhibited TGFβ-induced internalization of the TGFβ receptor without altering TGFβ-induced signaling and cellular responses. These conflicting results regarding the role of endocytosis in TGFβ-induced signaling and responses could be due to the different levels of dynamin K44A expression in the experimental systems used (Di

Guglielmo et al., 2003; Lu et al., 2002). To define the subcellular locus of TGFβ-induced signaling, we have determined the effects of several known inhibitors of clathrin-dependent endocytosis, including the dynamin inhibitor dynasore (Macia et al., 2006; Nankoe and Sever, 2006), on TGFβ-induced signaling and cellular responses. This approach is an alternative to methods dependent on overexpression of dynamin K44A that can yield results that vary depending on the level of expression.

Results

A number of compounds have been shown to inhibit clathrin-dependent endocytosis. These include methyl-β-cyclodextrin (β-CD) (Rodal et al., 1999), phenothiazines (Horwitz et al., 1981; Kuratomi et al., 1986; Salisbury et al., 1980), monodansylcadaverine (MDC) (Schlegel et al., 1982), chloroquine (Wang et al., 1993), monensin (Dickson et al., 1982), hyperosmotic sucrose (Hansen et al., 1993) and dynasore (Macia et al., 2006; Nankoe and Sever, 2006). β-CD inhibits clathrin-dependent endocytosis by selectively extracting cholesterol from the plasma membrane. Hydrophobic amines such as phenothiazines, MDC and chloroquine inhibit clathrin-dependent endocytosis by affecting the function of clathrin and clathrin-coated vesicles (Salisbury et al., 1980; Wang et al., 1993). Monensin is a monovalent ionophore that inhibits clathrin-dependent endocytosis by dissipating a proton gradient (Dickson et al., 1982). Hyperosmotic sucrose inhibits clathrin-dependent endocytosis by preventing clathrin and adaptors from interacting (Hansen et al., 1993). Dynasore is a cell-permeable inhibitor of dynamin GTPase activity that facilitates the formation of coated pits in the process of endocytosis (Macia et al., 2006; Nankoe and Sever, 2006).

If TGFβ-induced signaling occurs in endosomes, as reported previously (Di Guglielmo et al., 2003; Hayes et al., 2002), inhibitors of clathrin-dependent endocytosis would be expected to attenuate TGFβ-stimulated signaling such as Smad2 phosphorylation and nuclear localization (Heldin et al., 1997; Massague, 1998). To test this, Mv1Lu cells were pretreated with vehicle only or with clathrin-dependent endocytosis inhibitors at 37°C for 30 minutes and then stimulated with or without 100 pM TGFβ1. At the appropriate time-points, the relative levels of P-Smad2 in treated and stimulated cells were analyzed by quantitative western blot analysis using antibodies against P-Smad2 and Smad2. As shown in Fig. 1, β-CD, MDC, monensin and triflupromazine (TFP) enhanced TGFβ1-stimulated Smad2 phosphorylation in a time-dependent manner (Fig. 1Aa,Ba,Ca,Da, respectively). After stimulation of cells with TGFβ1 for 60 minutes, β-CD, MDC, monensin and TFP enhanced TGFβ1-stimulated Smad2 phosphorylation by ~1.5 to 2.5 fold (Fig. 1Ab,Bb,Cb,Db). To characterize further the effects of these inhibitors on Smad2 phosphorylation, Mv1Lu cells were pretreated with vehicle only or with several concentrations of the inhibitors at 37°C for 30 minutes and then stimulated with 100 pM of TGFβ1. After 30 minutes at 37°C, cell lysates were subjected to western blot analysis using antibodies against P-Smad2 and Smad2 followed by quantitation with densitometry. As shown in Fig. 1E-I, β-CD, TFP, monensin, chloroquine and dynasore enhanced TGFβ1-stimulated Smad2 phosphorylation in a concentration-dependent manner. β-CD at 10 mg/ml, TFP (24 μM), monensin (40 μM), chloroquine (200 μM) and dynasore (40 μM) enhanced TGFβ1-stimulated Smad2 phosphorylation by approximately two to three fold. To determine the effect of β-CD on TGFβ1-stimulated nuclear localization of P-Smad2, Mv1Lu cells were pretreated with vehicle only or with β-CD (10 mg/ml). After 1 hour at 37°C, cells were stimulated with TGFβ1 (10 pM) for 30 minutes. The nuclear localization of P-Smad2 was then analyzed by indirect fluorescent staining. As shown in Fig. 1J, β-CD and TGFβ1 together promoted nuclear localization of P-Smad2 (d), whereas each when applied on its own did not promote such localization (b and c).

The endocytosis inhibitors tested here have been shown to inhibit the pinching-off of endocytic vesicles from the plasma membrane (formation of endosomes) and arrest the endocytosis process at coated-pit stages (Rodal et al., 1999). This suggests that the coated-pit stages in the process of clathrin-dependent endocytosis might play important roles in mediating TGFβ-induced signaling. To define the coated-pit stages that are important in TGFβ1-induced signaling, we treated Mv1Lu cells with hyperosmotic sucrose (0.45 M) or β-CD and examined TGFβ1-stimulated Smad2 phosphorylation in these cells. Hyperosmotic sucrose is known to inhibit the formation of shallow coated pits (type 1 coated pits) or receptor clustering (Hansen et al., 1993). β-CD has been shown to inhibit progression from shallow coated pits (type 1 coated pits) to invaginated coated pits (type 2 coated pits) in the clathrin-dependent endocytosis process (Rodal et al., 1999). As shown in Fig. 1K, hyperosmotic sucrose inhibited TGFβ1-stimulated Smad2 phosphorylation in the cells treated with various concentrations of TGFβ1 (Fig. 1Ka,Kb). In cells stimulated with 100 pM TGFβ1, hyperosmotic sucrose attenuated Smad2 phosphorylation by ~60% (Fig. 1Kb). By contrast, β-CD enhanced TGFβ1-stimulated Smad2 phosphorylation at all concentrations of TGFβ1. It enhanced TGFβ1-stimulated Smad2 phosphorylation by approximately two to four fold when compared with the control (treatment without β-CD) (Fig. 1Ka,Kb). These results suggest that TGFβ-induced signaling occurs at the type 1 coated-pit stage.

TGFβ stimulates Smad2 phosphorylation by inducing association of TβR-I and SARA, which serves as an anchor for Smad2 (Tsukazaki et al., 1998; Xu et al., 2000), binding of Smad2 to SARA, and subsequent phosphorylation of Smad2 by TβR-I in the TβR-I–SARA–Smad2 complex. If clathrin-dependent endocytosis inhibitors enhance TGFβ-induced signaling (TGFβ-stimulated Smad2 phosphorylation) by increasing accumulation of TβR-I–TβR-II complexes at the coated pits, they should promote colocalization and accumulation of TβR-I and SARA at the plasma membrane. To test this, Mv1Lu cells were stimulated with 100 pM

Fig. 1. Enhancement of TGFβ-stimulated Smad2 phosphorylation and nuclear localization by clathrin-dependent endocytosis inhibitors in Mv1Lu cells. (A-I,K) Cells were pretreated with 10 mg/ml β-CD (A,K), 20 μM MDC (B), 40 μM monensin (C), 20 μM TFP (D) and 0.45 M sucrose (K) or several concentrations (as indicated) of β-CD (E), TFP (F), monensin (G), chloroquine (H) and dynasore (I), at 37°C for 1 hour. The cells were then stimulated with vehicle only or 100 pM TGFβ1 (A-I) or several concentrations (as indicated) of TGFβ1 (K). At various time-points, as indicated, at 37°C, cell lysates were analysed by 7.5% SDS PAGE followed by western blot analysis using antibodies against P-Smad2 and Smad2 and chemiluminescence development (a or top) and quantitation by densitometry (b or bottom). The data shown (a or top) are representative of three independent experiments. The relative level of P-Smad2 was expressed as arbitrary units (A.U.) (A,B,C,D,K) (b). The relative level of P-Smad2 in cells treated with TGFβ alone was taken as 100% of control (E-I, bottom). Experiments were performed in triplicate. The data are means ± s.d. Asterisks (*) indicate results significantly higher than that in cells treated with TGFβ alone; $P<0.001$. (J) Cells were pretreated with 10 mg/ml β-CD (c,d) at 37°C for 1 hour. The cells were then stimulated with vehicle only (a) or with 100 pM TGFβ (b,d). After 30 minutes at 37°C, the cells were fixed and subjected to indirect immunofluorescence staining using an antibody against P-Smad2. The data shown are representative of three independent experiments. Scale bar: 5 μm.

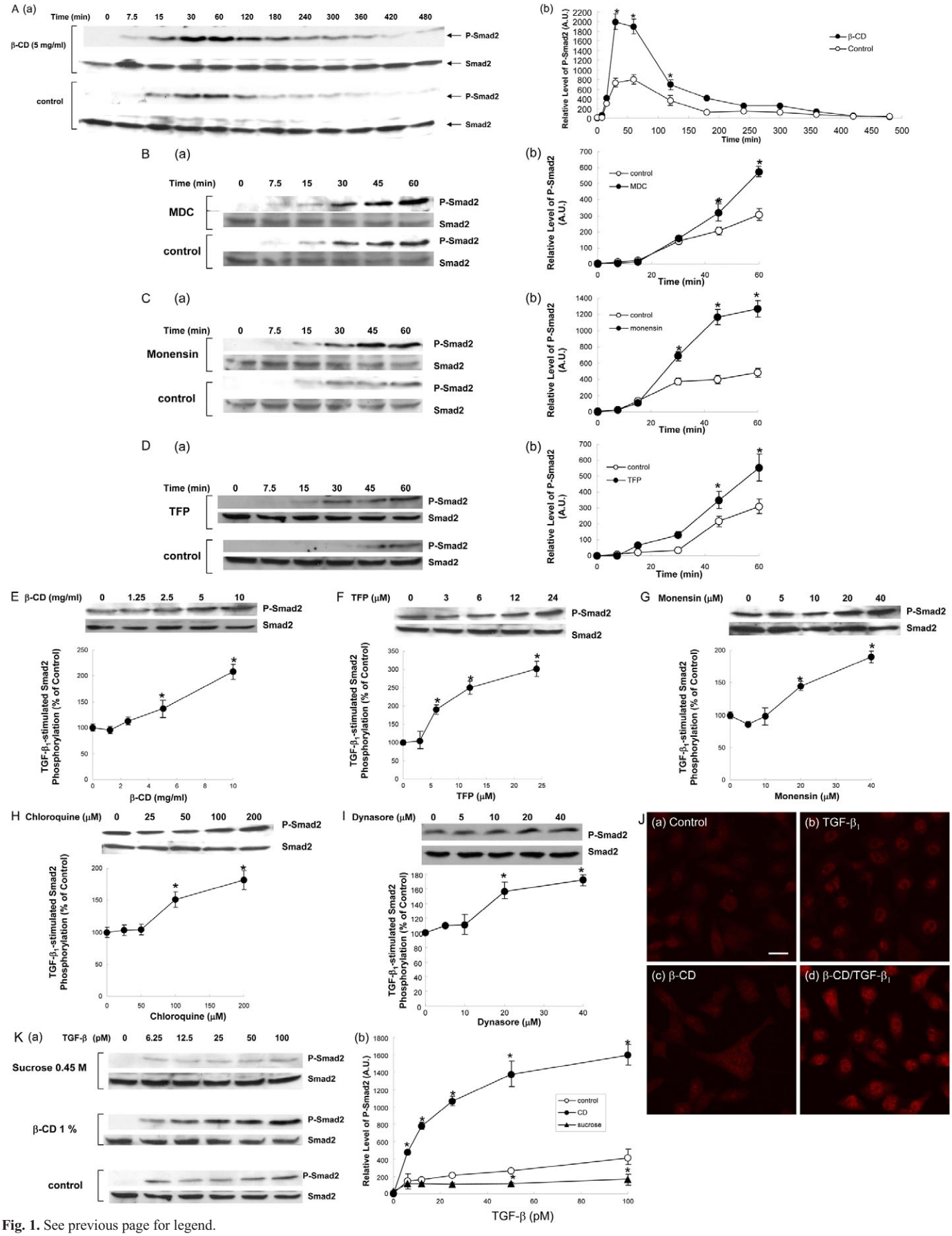

Fig. 1. See previous page for legend.

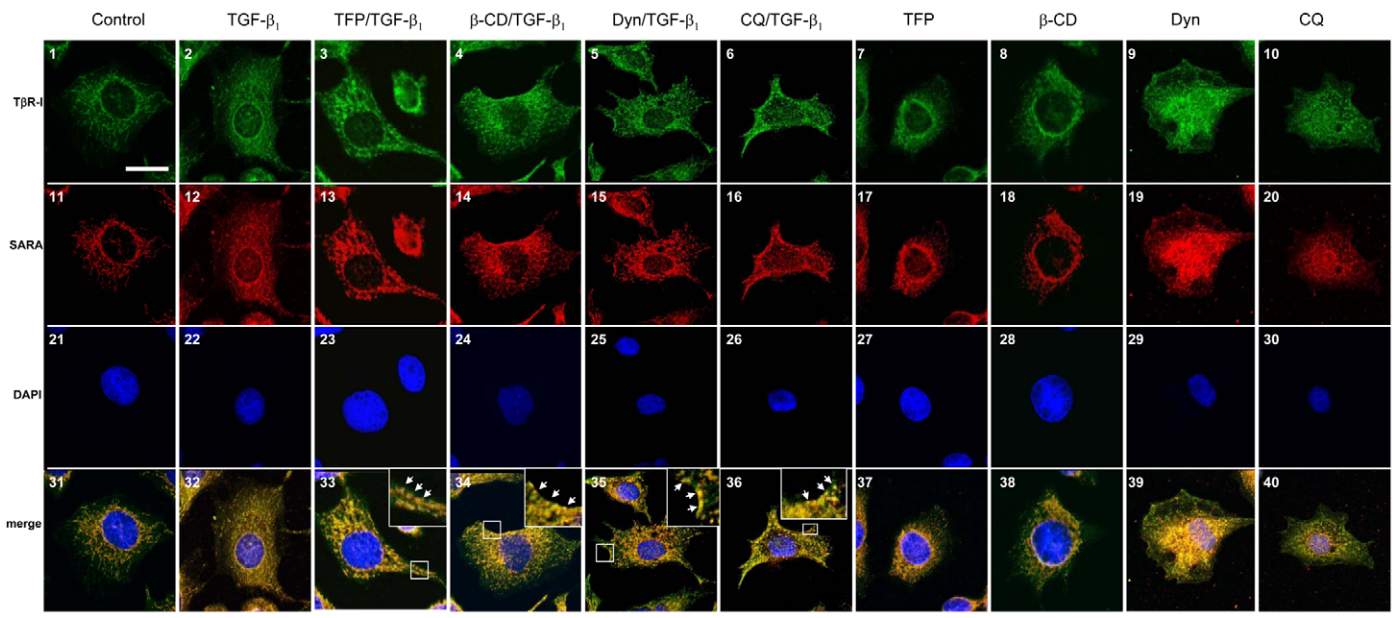

Fig. 2. Enhancement of colocalization and accumulation of SARA and TβR-I at the plasma membrane by inhibitors of clathrin-dependent endocytosis in Mv1Lu cells. Cells were pretreated with vehicle only or 20 μM TFP, 10 mg/ml β-CD, 40 μM dynasore (Dyn) and 200 μM chloroquine (CQ) at 37°C for 1 hour. Treated cells were then stimulated with and without 100 pM TGFβ1. After 30 minutes at 37°C, cells were fixed and analyzed by indirect immunofluorescence staining using antibody against TβR-I (panels 1-10) and SARA (panels 11-20); DAPI (nuclear) staining was also performed (panels 21-30). Merged staining is also shown (panels 31-40). Insets indicate the colocalization and accumulation of SARA and TβR-I at the plasma membrane.

TGFβ1. After 30 minutes at 37°C, cells were treated with the inhibitors and analyzed by immunofluorescence microscopy using antibodies against TβR-I and SARA. As shown in Fig. 2, TGFβ1 alone stimulated colocalization and accumulation of TβR-I and SARA in endocytic vehicles (endosomes) (Fig. 2, panel 32 versus panel 31) in Mv1Lu cells. This is consistent with the previous report that TGFβ enhances TGFβ receptor internalization (Lu et al., 2002). However, co-treatment of cells with TGFβ1 and TFP, β-CD, dynasore or chloroquine promoted colocalization and accumulation of TβR-I and SARA at the plasma membrane in these cells (Fig. 2, panels 33-36, inset). These endocytosis inhibitors (except dynasore) alone did not cause colocalization and accumulation of TβR-I and SARA at the plasma membrane (Fig. 2, panels 37-40 versus panel 31). Cells treated with dynasore alone exhibit moderate accumulation and colocalization of TβR-I and SARA (Fig. 2, panel 39). These results support the notion that clathrin-dependent endocytosis inhibitors enhance TGFβ-induced signaling (or TGFβ-stimulated Smad2 phosphorylation and nuclear localization) by promoting localization and accumulation of SARA–TGFβ receptor complexes at the plasma membrane or coated-pit stages.

The gene encoding PAI-1 is one of the most studied genes responsive to TGFβ stimulation (Heldin et al., 1997; Massague, 1998). The promoter region of this gene contains several Smad2/3 binding sites that have been used as TGFβ-responsive elements to enhance the expression of a reporter gene (Heldin et al., 1997; Massague, 1998). As clathrin-dependent endocytosis inhibitors are enhancers for TGFβ1-induced signaling, we determined the effects of these inhibitors on TGFβ1-stimulated expression of PAI-1 in Mv1Lu cells. Cells were pretreated with vehicle only and several concentrations of β-CD, thioridazine, TFP, MDC, monensin and chloroquine at 37°C for 1 hour. Cells were then stimulated with 50 pM TGFβ1. After 2 hours at 37°C, the relative levels of PAI-1 mRNA were analyzed by quantitative northern blot analysis (Fig. 3A,B) and real-time RT-PCR (Fig. 3C-G). As shown in Fig. 3, these inhibitors enhanced TGFβ1-stimulated expression in a concentration-dependent manner. β-CD at 10 mg/ml (A,C), 5 μM thioridazine (B), 6 μM TFP (D), 100 μM chloroquine (E), 10 μM monensin (F) and 100 μM MDC (G) enhanced TGFβ1-stimulated expression of PAI-1 by ~1.8 to 4.5 fold. As, among the inhibitors tested, only dynasore alone was found to stimulate PAI-1 expression, we determined the effects of several concentrations of dynasore with and without 50 pM TGFβ1 on PAI-1 expression in Mv1Lu cells. As shown in Fig. 3H, dynasore alone moderately stimulated PAI-1 expression. At 50 μM, dynasore enhanced TGFβ1-stimulated PAI-1 expression by approximately four to five fold in Mv1Lu cells. In separate experiments, the TGFβ-enhancing effect of dynasore was completely reversed by co-treatment of Mv1Lu cells and Mv1Lu cells stably expressing the luciferase reporter gene driven by the PAI-1 promoter (MLECs) (Chen et al., 2007) with 10 μM of SB 431542, a specific TβR-I (Alk5) kinase inhibitor, as determined by measuring PAI-1 mRNA with real-time RT-PCR (Fig. 3Ia) and by determining the luciferase activity (Fig. 3Ib), respectively. SB 431542 also completely reversed the TGFβ-mimicking effect of dynasore, as determined by measuring the luciferase activity in MLECs (Fig. 3Ib), suggesting that TβR-I signaling is responsible for mediating the inherent TGFβ-like activity of dynasore.

Suppressed TGFβ responsiveness in vascular cells has recently been found to play an important role in the pathogenesis of atherosclerosis induced by hypercholesterolemia (Chen et al., 2007; Chen et al., 2008). We have hypothesized that TGFβ enhancers such as inhibitors of clathrin-dependent endocytosis might ameliorate atherosclerosis caused by cholesterol-induced suppression of TGFβ responsiveness in vascular cells (Chen et al., 2007; Chen et al., 2008). To test our hypothesis, we treated hypercholesterolemic *ApoE*-null mice with dynasore (1 mg/kg body mass) through

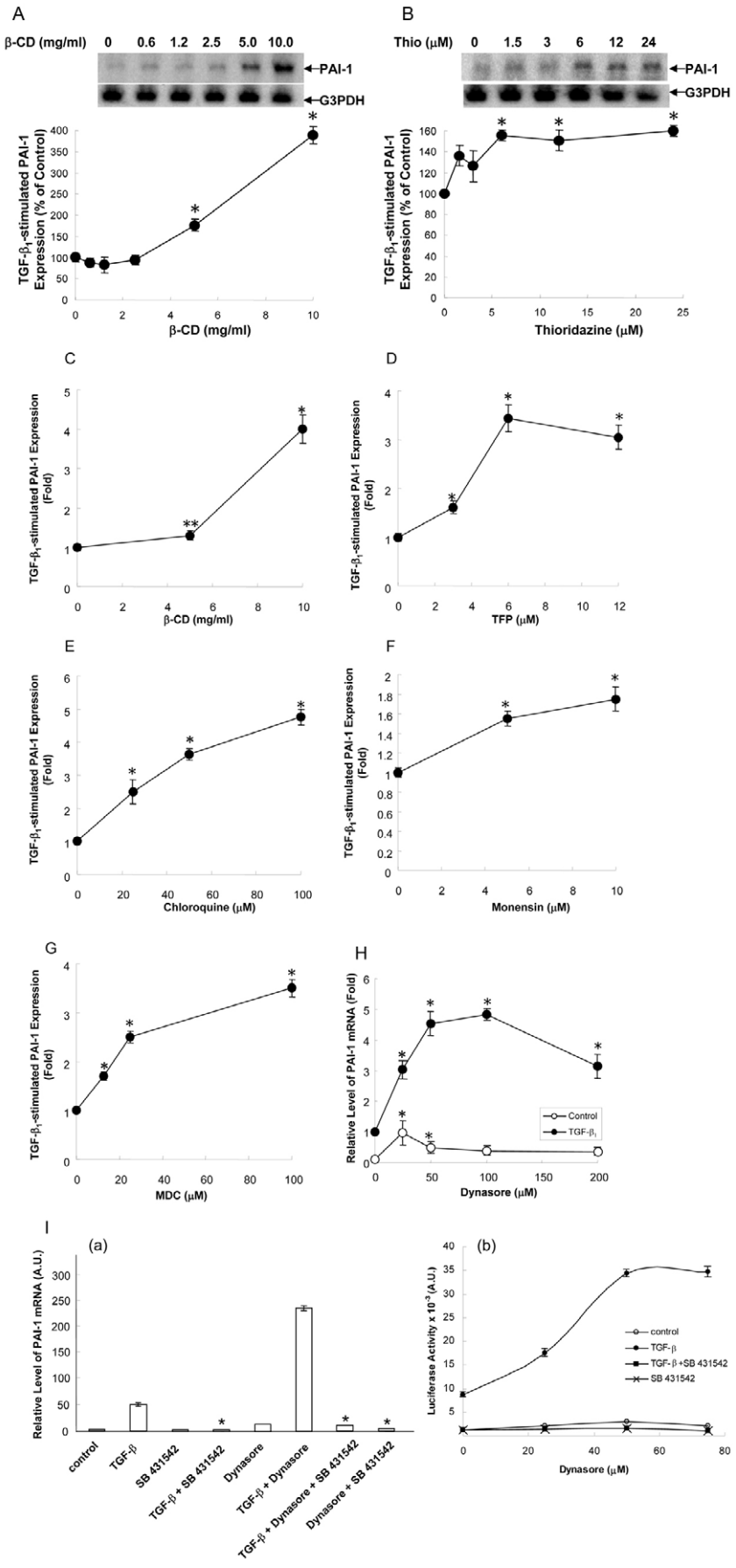

Fig. 3. Enhancement of TGFβ-stimulated expression of PAI-1 by inhibitors of clathrin-dependent endocytosis in Mv1Lu cells. (A-G) Mv1Lu cells were pretreated with vehicle alone or with several concentrations (as indicated) of β-CD (A,C), thioridazine (B), TFP (D), chloroquine (E), monensin (F) and MDC (G) at 37°C for 1 hour. Treated cells were then stimulated with 50 pM TGFβ1. After 2 hours at 37°C, the mRNAs encoding PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH, as control) in the cell lysates were analyzed by northern blot (top) and quantified using a PhosphorImager (bottom) (A,B) or real-time RT-PCR (C-G). The TGFβ-stimulated expression of PAI-1 in cells treated with vehicle only was taken as 100% (A,B) or one fold (C-G) of control. Experiments were carried out in triplicate. The data are means ± s.d. Asterisks indicate result significantly higher than control treated without the inhibitor (*, $P<0.001$; **, $P<0.05$). (H) Mv1Lu cells were pretreated with different concentrations (as indicated) of dynasore at 37°C for 1 hour and then stimulated with 50 pM TGFβ1. After 2 hours at 37°C, the mRNAs for PAI-1 and β-actin were quantitated by real-time RT-PCR. The relative mRNA level of PAI-1:β-actin in cells treated with TGFβ1 alone was taken as 1.0 (100%). Experiments were carried out in triplicate. The data are means ± s.d. Asterisk (*) indicates result significantly higher than control treated without the inhibitor, $P<0.001$. (Ia) Mv1Lu cells were pretreated with SB 431542 (10 μM) at 37°C for 1 hour and dynasore (50 μM) at 37°C for 0.5 hours, alone or together, and then stimulated with and without 20 pM TGFβ1. After 2 hours at 37°C, the mRNAs for PAI-1 and β-actin were quantitated by real-time RT-PCR. The relative mRNA level of PAI-1:β-actin in cells treated with vehicle only (control) was taken as 1.0. Experiments were carried out in triplicate. The data are means ± s.d. Asterisks (*) indicates result significantly lower than cells treated with the same agent(s) but without SB 431542; $P<0.001$. (Ib) Mv1Lu cells stably expressing the luciferase reporter gene driven by the PAI-1 promoter (MLECs-clone 32) were pretreated with SB 431542 (10 μM) at 37°C for 1 hour and several concentrations (as indicated) of dynasore at 37°C for 0.5 hours, alone or together, and then stimulated with and without 20 pM TGFβ1 at 37°C for 6 hours. The stimulated cells were lysed in 100 μl of lysis buffer. The cell lysates were assayed using a luciferase kit (Promega). The luciferase activity (A.U.) in treated and stimulated cells was determined. Experiments were carried out in triplicate. The data are means ± s.d.

intraperitoneal administration every 2 days for 8 weeks. We chose dynasore for several reasons. These were: (1) among inhibitors that we tested, dynasore is one of the most potent TGFβ enhancers in Mv1Lu cells and other cell types, including bovine aortic endothelial cells (BAEC cells) and Chinese hamster ovary (CHO) cells (C-L.C., S.S.H. and J.S.H., unpublished). At 50 μM, it enhances TGFβ-stimulated expression of PAI-1 in these cell types by approximately four to five fold; (2) dynasore is the only inhibitor tested that alone is capable of stimulating PAI-1 expression and colocalization and accumulation of P-Smad2 and SARA at the plasma membrane. It is a TGFβ enhancer as well as a TGFβ mimetic; and (3) no apparent macroscopic or microscopic abnormality has been detected in the liver, heart, lung and kidney of wild-type mice following intraperitoneal administration of dynasore (1 mg/kg body mass) every 2 days for 8 weeks. As shown in Fig. 4A,B, many macrophages or foam cells were attached to the endothelium of coronary arteries and descending aorta in hypercholesterolemic *ApoE*-null mice treated without dynasore (Fig. 4Ac,Ba). These mice also exhibited thickening of aortic valves (Fig. 4Bb). By contrast, very few macrophages or foam cells were found in the endothelium of the coronary arteries and descending aorta in hypercholesterolemic *ApoE*-null mice treated with dynasore (Fig. 4Ad,Bc). These dynasore-treated mice showed reduced thickening of aortic valves (Fig. 4Bd versus Fig. 4Bb). Quantitative analysis of the atherosclerotic lesion areas revealed that dynasore significantly reduced the lesion/media area ratio from 21% to 13.8% (Fig. 4C). Dynasore did not affect the plasma levels of cholesterol in *ApoE*-null mice (460±50 mg/dl in *ApoE*-null mice treated with dynasore versus 470±61 mg/dl in *ApoE*-null mice treated without dynasore). In wild-type mice treated with either vehicle alone or

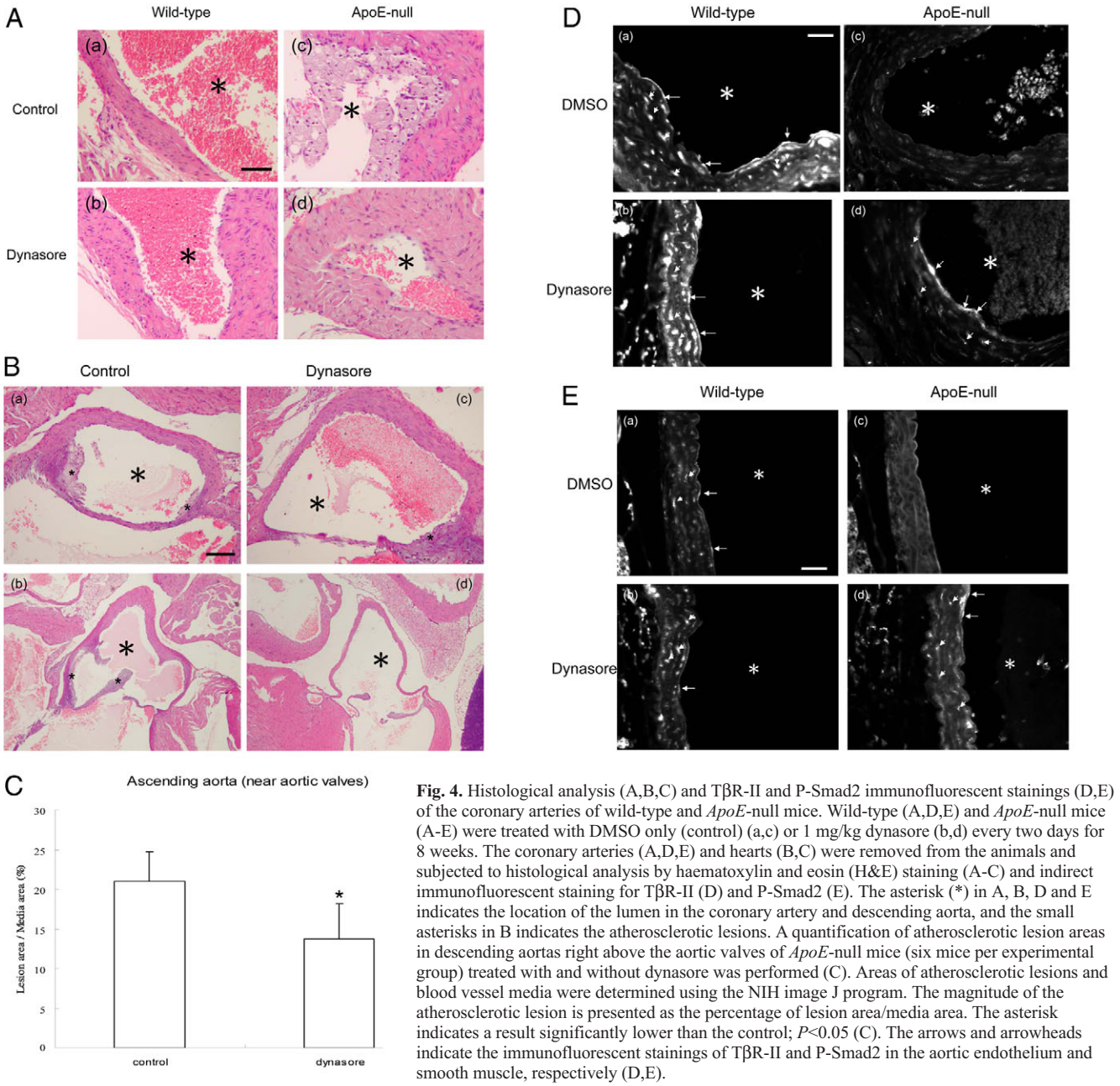

Fig. 4. Histological analysis (A,B,C) and TβR-II and P-Smad2 immunofluorescent stainings (D,E) of the coronary arteries of wild-type and *ApoE*-null mice. Wild-type (A,D,E) and *ApoE*-null mice (A-E) were treated with DMSO only (control) (a,c) or 1 mg/kg dynasore (b,d) every two days for 8 weeks. The coronary arteries (A,D,E) and hearts (B,C) were removed from the animals and subjected to histological analysis by haematoxylin and eosin (H&E) staining (A-C) and indirect immunofluorescent staining for TβR-II (D) and P-Smad2 (E). The asterisk (*) in A, B, D and E indicates the location of the lumen in the coronary artery and descending aorta, and the small asterisks in B indicates the atherosclerotic lesions. A quantification of atherosclerotic lesion areas in descending aortas right above the aortic valves of *ApoE*-null mice (six mice per experimental group) treated with and without dynasore was performed (C). Areas of atherosclerotic lesions and blood vessel media were determined using the NIH image J program. The magnitude of the atherosclerotic lesion is presented as the percentage of lesion area/media area. The asterisk indicates a result significantly lower than the control; $P<0.05$ (C). The arrows and arrowheads indicate the immunofluorescent stainings of TβR-II and P-Smad2 in the aortic endothelium and smooth muscle, respectively (D,E).

dynasore, the coronary arteries and descending aorta exhibited normal morphology (Fig. 4Aa,Ab and data not shown, respectively). The plasma levels of cholesterol in wild-type mice treated with either vehicle alone or dynasore were 120±10 and 125±20 mg/dl, respectively.

As hypercholesterolemia has been shown to downregulate the expression of TβR-II and phosphorylated Smad2 (P-Smad2) in the aortic endothelium of *ApoE*-null mice (Chen et al., 2008), we examined the expression of TβR-II and P-Smad2 in the animals treated with dynasore. As shown in Fig. 4D,E, the TβR-II and P-Smad2 stainings were found in the aortic endothelium as well as in the smooth muscle of coronary arteries in wild-type mice treated with either vehicle alone or dynasore (Fig. 4Da/Db and Fig. 4Ea/Eb, respectively). However, no TβR-II or P-Smad2 staining was found in the aortic endothelium and smooth muscle of coronary arteries in *ApoE*-null mice treated with vehicle alone (Fig. 4Dc,Ec). Dynasore treatment appeared to restore or enhance the TβR-II and P-Smad2 stainings in the aortic endothelium and smooth muscle of coronary arteries in the animals (Fig. 4Dd,Ed, respectively). Interestingly, enhanced stainings of TβR-II and P-Smad2 were also found in the endothelium and smooth muscle of coronary arteries in wild-type mice treated with dynasore when compared with those treated with DMSO alone (vehicle) (Fig. 4Db versus Fig. 4Da and Fig. 4Eb versus Fig. 4Ea, respectively). These results suggest that dynasore is effective in ameliorating atherosclerosis, at least in part, by counteracting the downregulation of TβR-II and P-Smad2 expression caused by hypercholesterolemia in the aortic endothelium of *ApoE*-null mice (Chen et al., 2008). These results also suggest that dynasore is capable of enhancing TGFβ signaling in coronary arteries in wild-type mice.

Discussion

Clathrin-dependent endocytosis inhibitors are known to inhibit the endocytosis process at different steps. Hyperosmotic sucrose blocks formation of type 1 coated pits (Mousavi et al., 2004; Rodal et al., 1999). β-CD and dynasore inhibit progression from type 1 coated pits to type 2 coated pits (Nankoe and Sever, 2006; Rodal et al., 1999). Phenothiazines, MDC and chloroquine inhibit the progression from type 2 coated pits to type 3 coated pits (Schlegel et al., 1982; Wang et al., 1993). Monensin and dynasore inhibit progression from type 3 coated pits to coated vesicles (Dickson et al., 1982; Nankoe and Sever, 2006). All of the inhibitors tested, except hyperosmotic sucrose, enhanced TGFβ-induced signaling and responses. As these inhibitors arrest endocytosis at coated-pit stages, this suggests that TGFβ-induced signaling mainly occurs at coated-pit stages. This suggestion is supported by the observation that hyperosmotic sucrose inhibits the formation of type 1 coated pits and attenuates TGFβ-induced signaling and responses. As dynamin is required for both processes leading to formation of type 2 coated pits and coated vesicles, specific inhibition of dynamin by dynasore was expected to block clathrin-dependent endocytosis at these two steps and increase accumulation of coated pits. This would explain why dynasore is a more potent inhibitor for clathrin-dependent endocytosis and a more potent TGFβ enhancer than other inhibitors, such as phenothiazines, MDC and monensin, all of which inhibit endocytosis at a single step.

All of these clathrin-dependent endocytosis inhibitors inhibit internalization of cell-surface-bound TGFβ (supplementary material Fig. S1). All inhibitors except hyperosmotic sucrose enhance colocalization and accumulation of TβR-I and SARA at the plasma membrane, as demonstrated by immunofluorescence microscopy. This is consistent with their reported activity in arresting the process of endocytosis at the type 1, type 2 or type 3 coated-pit stages (Rodal et al., 1999; Zwaagstra et al., 2001). It has been demonstrated that overexpression of dynamin K44A enhances accumulation of TβR-I at the plasma membrane (Lu et al., 2002). Lu et al. (Lu et al., 2002) reported that overexpression of dynamin K44A inhibits internalization of cell-surface TβR-I but does not affect TGFβ-stimulated cellular responses. In fact, their data shows that overexpression of dynamin K44A enhances TGFβ-stimulated responses by approximately two fold, when compared with controls, in their experimental system. We suggest that overexpression of wild-type dynamin is not an appropriate control for overexpression of dynamin K44A in such data (Lu et al., 2002). Overexpression of wild-type dynamin might enhance TGFβ-stimulated cellular responses (expression of 3TP-luciferase and ARE-luciferase) by promoting endocytosis and thus increasing formation of coated pits (Lu et al., 2002). By contrast, overexpression of K44A dynamin might enhance TGFβ-stimulated cellular responses by arresting endocytosis at the coated-pit stages (Lu et al., 2002)

We recently found that cholesterol suppresses TGFβ responsiveness in cultured cells and in the aortic endothelium of *ApoE*-null mice with hypercholesterolemia (Chen et al., 2007; Chen et al., 2008). As accumulating evidence indicates that TGFβ in blood is a protective cytokine for atherosclerosis (Metcalfe and Grainger, 1995), this suggests that hypercholesterolemia causes atherosclerosis, at least in part, by suppressing TGFβ responsiveness (Chen et al., 2007; Chen et al., 2008). Here, we demonstrate that dynasore, a potent TGFβ enhancer, effectively ameliorates atherosclerosis in *ApoE*-null mice, presumably by counteracting the suppressed TGFβ responsiveness caused by hypercholesterolemia (Chen et al., 2007; Chen et al., 2008). As the downregulation of TGFβ levels and/or TGFβ responsiveness has been implicated in other disease processes, such as autoimmune disease (Li and Flavell, 2008), potent TGFβ-enhancers such as dynasore or dynasore-like compounds are potential therapeutic compounds for treating such diseases.

Materials and Methods

Cell-surface-bound ^{125}I-labelled TGFβ internalization

Mv1Lu cells grown to confluence on 24-well culture dishes were treated with 10 mg/ml β-CD, 20 μM MDC, 40 μM monensin, 20 μM thioridiazine, 25 μM nystatin and 200 μM chloroquine in serum-free DMEM (0.25 ml/well) at 37°C for 1 hour. Treated cells were washed with cold binding buffer and incubated with 100 pM ^{125}I-labeled TGFβ1 in the presence and absence of a 100-fold excess of unlabeled TGFβ1 (to determine nonspecifically internalized and total internalized ^{125}I-labeled TGFβ1, respectively) in binding buffer containing 0.2% bovine serum albumin (BSA) at 4°C for 2 hours. After ^{125}I-labeled TGFβ1 binding, cells were washed with cold DMEM and incubated in DMEM at 37°C for various time periods (0 to 10 minutes). Cells were then cooled to 4°C and treated with trypsin (2 mg/ml) at 4°C for 2 hours. After trypsin digestion and centrifugation, cells were solubilized with 0.2 M NaOH and the cell-associated radioactivity (internalized ^{125}I-labeled TGFβ1) was determined by a γ-counter. Specifically internalized ^{125}I-labeled TGFβ1 was estimated by subtracting nonspecifically internalized ^{125}I-labeled TGFβ1 from total internalized ^{125}I-labeled TGFβ1. In the experiments, MDC and nystatin were prepared in a stock solution containing DMSO as a solvent vehicle. Thioridazine was solubilized in ethanol. The final concentrations of DMSO and ethanol in the medium were 0.2%. The inhibitor compounds (all from Sigma) were present throughout ^{125}I-labeled TGFβ1 binding and internalization experiments.

Western blot analysis for Smad2 phosphorylation

Mv1Lu cells grown to near confluence on 12-well culture dishes were treated with 10 mg/ml β-CD, 20 μM MDC, 40 μM monensin, and 20 μM TFP in serum-free DMEM (0.5 ml/well) at 37°C for 1 hour. The final concentrations of ethanol and DMSO in the medium were 0.2%. The treated cells were incubated with 50 pM TGFβ1 at 37°C for various periods of time. Treated cells were lysed by SDS sample buffer, and cell lysates with equal amounts of protein (200 μg) were analyzed by 7.5% SDS–PAGE followed by western blotting using anti-Smad2 and anti-P-Smad2

(Cell Signaling). The relative levels of Smad2 and P-Smad2 were determined by densitometry.

P-Smad2 nuclear localization

Mv1Lu cells grown to 50% confluence on glass cover slips were treated with 10 mg/ml β-CD in serum-free DMEM at 37°C for 1 hour. Treated cells were incubated with 20 pM TGFβ1 at 37°C for 30 minutes. After TGFβ1 stimulation, cells were fixed in methanol (–20°C) for 15 minutes, washed with PBS and blocked with 0.2% gelatin in phosphate-buffered saline (PBS) for 1 hour. Cells were then incubated with rabbit antibody to P-Smad2 (Cell Signaling) at 1:100 dilution in a humidified chamber at 4°C overnight. After extensive washing, cells were incubated with rhodamine-conjugated mouse anti-rabbit IgG at a 1:50 dilution for 1 hour. The subcellular localization of P-Smad2 was determined by examination under a fluorescence microscope.

Colocalization of SARA and TβR-I

Mv1Lu cells grown to 50% confluence on glass cover slips were treated with 20 μM TFP, 10 mg/ml β-CD, 40 μM dynasore (Dyn) and 200 μM chloroquine (CQ) in serum-free DMEM at 37°C for 1 hour. Treated cells were incubated with 100 pM TGFβ1 at 37°C for 30 minutes. After TGFβ1 stimulation, cells were fixed in methanol (–20°C) for 15 minutes, washed with PBS and blocked with 0.2% gelatin in PBS for 1 hour. Cells were then incubated with rabbit antibody against TβR-I (Santa Cruz Biotechnology) and goat antibody against SARA (Santa Cruz Biotechnology) at a 1:100 dilution in a humidified chamber at 4°C overnight. After extensive washing, cells were incubated with a rhodamine-conjugated donkey anti-goat IgG antibody and FITC-conjugated mouse anti-rabbit IgG antibody at a 1:50 dilution for 1 hour. Images were acquired using a Leica TCS SP confocal microscope (Leica Microsystems, Heidelberg, Germany). The measurement of colocalization rate was analyzed using a Leica application suite.

Luciferase activity assay

Mv1Lu cells stably expressing the luciferase reporter gene driven by the PAI-1 promoter (MLECs-Clone 32) (Chen et al., 2007) grown to near-confluence on 12-well dishes were treated at 37°C with 10 μM SB 431542 for 1 hour and/or several concentrations of dynasore for 0.5 hours. Treated cells were further incubated with 20 pM TGFβ at 37°C for 6 hours and lysed in 100 μl of lysis buffer (Promega). The cell lysates (~20 μg protein) were then assayed using the luciferase kit from Promega.

Northern blot analysis

Cells grown to confluence on 12-well culture dishes in serum-free DMEM were treated with several concentrations of thioridazine, β-CD, TFP, chloroquine, MDC and monensin at 37°C for 1 hour. The treated cells were then incubated with 20 pM TGFβ1 at 37°C for 2 hours. The transcripts of PAI-1 and G3PDH (as a control) in cell lysates were analyzed by northern blotting and quantified by using a PhosphorImager.

Histological analysis

Female *ApoE*-null mice and wild-type mice (C57BL/6; 8 weeks old) were subdivided into four groups (six mice for each experimental group) and fed normal chow diet throughout the study. *ApoE*-null mice and wild-type mice were injected intraperitoneally with vehicle (DMSO) alone or dynasore (1 mg/kg), four times per week. At the end of the treatment period (16 weeks), all animals were sacrificed by administration of CO_2. Blood samples were collected by cardiac puncture at the time of sacrifice. The coronary arteries and hearts were removed and fixed in formalin–PBS for histological analysis with H&E staining and immunofluorescent staining using anti-P-Smad2 (Cell Signaling). Atherosclerotic lesions in ascending aorta near the heart valve were quantified as described previously (Bourdillon et al., 2006). The areas of atherosclerotic lesions and blood vessel media were determined using the NIH image J program. The magnitude of atherosclerotic lesions is presented as the percentage of lesion area/media area.

Quantitative analysis of PAI-1 mRNA (relative to β-actin mRNA) by real-time RT-PCR

Mv1lu cells were treated with several concentrations of TFP, β-CD, dynasore, monensin, MDC and chloroquine with and without 5 μM SB 431542, a specific TβR-I (Alk5) kinase inhibitor (Tocris Bioscience, MO) in serum-free DMEM at 37°C for 1 hour. After stimulation of the cells with 50 pM TGFβ1 at 37°C for 2 hours, RNAs from treated and untreated cells were isolated using the Trizol B (Teltext, TX) according to the manufacturer's instructions. cDNAs were made from the isolated RNAs using MuLV reverse transcriptase (Applied Biosystems) and 1 μg RNA. The reverse transcription reaction was performed under the following conditions: 42°C for 15 minutes, 99°C for 5 minutes and 4°C for 5 minutes. The SYBR green master mix was used with 200 nM of each primer. The real-time PCR was performed at 2 minutes at 94°C for one cycle followed by 1 minute at 94°C, 0.45 minutes at 60°C and 1 minute at 72°C for 35 cycles using a Bio-Rad Chrom 4 Thermocycler. The values of each experimental condition were normalized to the level of β-actin in a parallel sample. The primer sequences used were as follows: PAI-1 primer forward: 5′-GCCC-TACTTCTTCAGGCTGTTC-3′; PAI-1 reverse: 5′-GAACAGCCTGAAGAAG-TAGGGC-3′; β-actin forward: 5′-AGCCATGTACGTAGCCATCCAGGCTC-3′; and β-actin reverse: 5′-TGGGTACATGGTGGTACCACCAGACA-3′.

Clathrin-dependent endocytosis inhibitors inhibit internalization of cell-surface-bound TGFβ in Mv1Lu cells

As clathrin-dependent endocytosis is known to be involved in TGFβ-induced signaling, we wished to determine the effects of known inhibitors of clathrin-dependent endocytosis on internalization of cell-surface-bound TGFβ in Mv1Lu cells. Mv1Lu cells are a standard model cell system for investigating cellular responses to TGFβ (Chen et al., 2008). Mv1Lu cells were incubated with 100 pM ^{125}I-labeled TGF-β1 (Huang et al., 2003) in the presence and absence of a 100-fold excess of unlabeled TGFβ1 at 0°C for 2.5 hours. After washing, cells were warmed to 37°C. After various time-points, cells were cooled on ice and treated with trypsin to remove non-internalized ^{125}I-labeled TGFβ1. The trypsin-resistant cell-associated radioactivities obtained from cells incubated in the presence and absence of excess unlabeled TGFβ1 were taken as indicating nonspecifically internalized and total internalized ^{125}I-labeled TGFβ, respectively. The specifically internalized ^{125}I-labeled TGFβ1 was estimated by subtracting nonspecifically internalized ^{125}I-labeled TGFβ1 from total internalized ^{125}I-labeled TGFβ1. The trypsin-resistant assay is commonly used for measuring internalized ligands or receptors in cells (Boensch et al., 1999). An acid-wash assay was previously used for determining internalized TGFβ but did not appear to be able to detect the inhibitory effect on clathrin-dependent endocytosis of dynamin dominant-negative mutant K44A and clathrin-dependent endocytosis inhibitors such as chloroquine and MDC (Zwaagstra et al., 2001). This might have been due to a nonspecific effect of the acid treatment such as increased TGFβ binding to cell-surface acidic pH binding sites (Ling et al., 2004).

As shown in supplementary material Fig. S1, β-CD inhibited ^{125}I-labeled TGFβ1 internalization by ~50-70% after 4 or 5 minutes incubation at 37°C, whereas triflupromazine (TFP), thioridazine and MDC inhibited ^{125}I-labeled TGFβ1 internalization by ~40-50% after 4- or 5-minute periods of incubation (supplementary material Fig. S1A,B). In the negative-control experiments, nystatin and colchicine did not significantly affect ^{125}I-labeled TGFβ1 internalization (supplementary material Fig. S1C,D). Nystatin is known to deplete cholesterol from lipid rafts/caveolae and enhances TGFβ-stimulated cellular responses. Colchicine enhances TGFβ-stimulated cellular responses by releasing Smad proteins from Smad-protein–microtubule complexes (Dong et al., 2000). Neither nystatin nor colchicine affected ^{125}I-labeled TGFβ1 internalization. β-CD is more potent than nystatin in depleting cholesterol from the plasma membrane and inhibits both caveolin- and clathrin-dependent endocytosis. Other known clathrin-dependent endocytosis inhibitors such as monensin, chloroquine, hyperosmotic sucrose and dynasore also inhibited cell-surface ^{125}I-labeled TGFβ1 internalization (supplementary material Fig. S1E-H). Monensin and chloroquine inhibited ^{125}I-labeled TGFβ internalization by ~50% and ~85%, respectively, after 8- or 10-minute incubations at 37°C (supplementary material Fig. S1E,F), whereas hyperosmotic sucrose and dynasore inhibited cell-surface TGFβ1 internalization by ~80% after a 5-minute incubation (supplementary material Fig. S1G,H). Hyperosmotic sucrose, dynasore, β-CD and chloroquine appeared to be more potent in inhibiting ^{125}I-labeled TGFβ internalization than other inhibitors tested.

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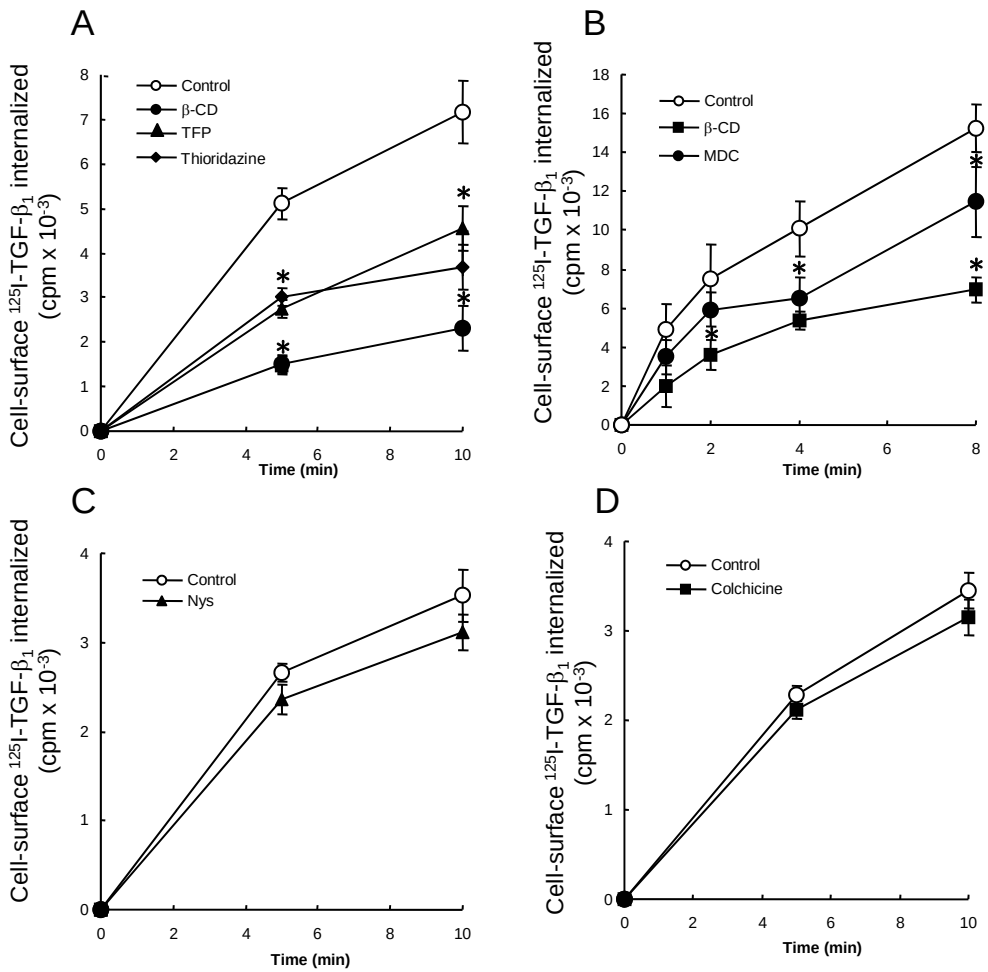
Supplementary

Fig 1A,B,C,D



Supplementary
Fig. 1E,F,G,H