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Myosin Mediates Contractile Force Generation by Hepatic Stellate Cells in Response to Endothelin-1

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Key Words
Actin · Myosin regulatory light chain · Phosphorylation · Signal transduction

Abstract
Although endothelin-1-stimulated contractile force generation by stellate cells is believed to play an important role in hepatic pathophysiology, the molecular signals that mediate this process are incompletely understood. The aim of this study was to test the hypothesis that myosin mediates the contractile force generated by stellate cells in response to endothelin-1. Contractile force generation by primary and immortalized stellate cells was directly and quantitatively measured. Myosin phosphorylation and reorganization, and actin stress fiber formation were investigated in immortalized stellate cells. Endothelin-1 stimulated a rapid and robust generation of contractile force by primary and immortalized stellate cells with a similar dose dependence. Myosin phosphorylation, actin stress fiber assembly, and reorganization of myosin to stress fibers were induced by concentrations of endothelin-1 that also stimulated stellate cell contraction. BQ-123, a selective endothelin receptor antagonist, inhibited myosin phosphorylation and contractile force generation. Y-27632, which selectively inhibits rho-associated kinase, also blocked endothelin-1-stimulated myosin phosphorylation and contractile force generation with a similar dose dependence. These results suggest that endothelin-1-stimulated contractile force generation by stellate cells is mediated by myosin.

The contraction of hepatic stellate cells in response to endothelin-1 is believed to contribute to critical processes in the liver including the regulation of sinusoidal resistance and the development of fibrosis [4, 11, 13, 23]. However, the signal transduction mechanisms that couple endothelin-1 to the generation of contractile force by stellate cells have not been clearly defined, in part because it has heretofore not been possible to directly measure the magnitude of the forces generated by the contraction of this cell type. In fact, to our knowledge, the endothelin-1 dose dependence for the magnitude of contractile force generated by stellate cells has not been reported previously. Nevertheless, it has been proposed that the contractile force generated by stellate cells is powered by the myosin II motor protein complex (myosin) [6] in the same way as it is in smooth muscle cells. In smooth muscle, contrac-

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tion is driven by interactions between myosin, which is activated by phosphorylation of its regulatory component, the myosin regulatory light chain (MRLC), and bundles of polymerized actin within the cytoplasm [15]. A role for myosin in the generation of contractile force by stellate cells is supported by the observation that in this cell type both the assembly of actin stress fibers, an anatomic correlate of contraction [7, 22], and collagen lattice shrinkage [7] are associated with an increase in myosin phosphorylation. On the other hand, contractile force generation by certain non-muscle cell types, including fibroblasts, may occur independent of myosin phosphorylation [10, 14]. In this study, we tested the hypothesis that myosin mediates the contractile force generated by stellate cells in response to endothelin-1 using a recently developed method for directly quantitating stellate cell contraction [18]. Our results show that endothelin-1 stimulates a rapid and dose-dependent increase in stellate cell contractile force in both primary cultures of stellate cells and immortalized stellate cells. Moreover, the data presented here suggest that endothelin-1 induces contractile force generation by stellate cells through the activation of myosin.

Methods

Cell Culture

Rat stellate cells, isolated as previously described [3, 19], were obtained from the Non-Parenchymal Liver Cell Core Facility of the Research Center for Alcoholic Liver and Pancreatic Diseases. Prior to use in the described experiments, isolated stellate cells were grown in primary culture for 10 days to permit activation [22]. An SV40-immortalized rat hepatic stellate cell line, previously shown to demonstrate many features of rat stellate cells in primary culture [2, 8, 9, 20], was obtained from Dr. S. Friedman and used during passages 8–14. Primary and immortalized stellate cells were cultured on Primaria-treated tissue culture dishes (Becton-Dickinson, Lincoln Park, N.J., USA) in Dulbecco's high glucose medium supplemented with 2% *L*-glutamine, 1% nonessential amino acids, 0.5% sodium pyruvate, 1% penicillin and streptomycin, and 10% fetal bovine serum. With the exception of fetal bovine serum (Sigma-Aldrich, St. Louis, Mo., USA), cell culture products were from Irvine Scientific (Santa Ana, Calif., USA). Cells were grown at 37°C in a humidified 10% CO_2 incubator. Medium was exchanged every 2–3 days.

Reagents

Endothelin-1 was purchased from Calbiochem-Novabiochem (San Diego, Calif., USA), sarafotoxin S6C (sarafotoxin) and BQ-123 from Peninsula Laboratories (San Carlos, Calif., USA), and Y-27632 [R-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] was a gift of Welfide (Osaka, Japan).

Experiments were performed in a physiological salt solution (135 m*M* NaCl, 5 m*M* KCl, 0.8 m*M* $MgCl_2$, 1.2 m*M* $CaCl_2$, 0.8 m*M* NaH_2PO_4, 5 m*M* glucose, and 10 m*M* HEPES; pH 7.4), except as noted.

Quantitation of Stellate Cell Contractile Force Generation

The magnitude of contractile force generation by stellate cells was directly measured as described [18]. Solid, but elastic collagen gels were formed containing either ~500,000 stellate cells in primary culture per gel or ~600,000 immortalized stellate cells per gel. After 3 days at 37°C in 10% CO_2 and media containing 10% FBS, the gels containing stellate cells were attached to an isometric force transducer and immersed in an organ bath containing physiological salt solution at 37°C for 1 h prior to stimulation to ensure a stable baseline tension. Experimental agents were added directly to the organ bath. BQ-123 and Y-27632 were added 10 and 30 min prior to stimulation with agonists, respectively. Changes in isometric tension were recorded digitally.

Fluorescent Staining of Polymerized Actin and Myosin

Fluorescent staining was performed as previously described [17, 22]. Stellate cells, cultured for 1 day at low density on collagen-treated glass coverslips, were placed in physiological salt solution for 3 h prior to experimentation. Stellate cells were treated with endothelin-1 or carrier for 15 min prior to fixation. Polymerized actin was stained with rhodamine-labeled phalloidin (Molecular Probes, Eugene, Oreg., USA), which binds to polymerized actin with high (nanomolar) specificity. Myosin was labeled with an antibody directed against MRLC [17] and then stained with a fluorescein-conjugated secondary antibody. Using an epifluorescent microscope, polymerized actin was visualized with a standard rhodamine filter, and myosin was visualized with a standard fluorescein filter (Chroma, Brattleboro, Vt., USA). The intensity of the light emitted by rhodamine using the fluorescein filter set was approximately 2.5% of the intensity of light emitted by rhodamine using the rhodamine filter set. Similarly, the intensity of the light emitted by fluorescein using the rhodamine filter set was approximately 2.5% of the intensity of light emitted by fluorescein using the fluorescein filter set. In contrast, in dual labeling experiments (i.e. polymerized actin and myosin), the light emitted using the fluorescein filter set (i.e. myosin) was consistently greater than 35% of the light emitted using the rhodamine filter set (i.e. polymerized actin). This indicated that in dual labeling experiments, the light emitted using the fluorescein filter set was in fact due to myosin staining rather than to unfiltered light resulting from the staining of polymerized actin. To minimize observer bias, a blinded investigator performed the experimental analysis.

Determination of Myosin Phosphorylation

Myosin phosphorylation was determined as previously described [21, 22, 24]. Unphosphorylated, monophosphorylated, and diphosphorylated forms of MRLC were separated by urea/glycerol-acrylamide gel electrophoresis. Immunoblot was performed with an antibody directed against MRLC. Bands representing phosphorylated and unphosphorylated forms of myosin were quantitated by densitometry. Myosin phosphorylation is presented as the sum of mono- and diphosphorylated myosin as a percentage of total myosin.

Statistics

Data are presented as means ± SEM. t tests for unpaired data were used to compare means. Results were considered significant if $p < 0.05$.

Results

Endothelin-1 Stimulates Contractile Force Generation by Stellate Cells

Endothelin-1 induced a rapid and substantial increase in contractile force by stellate cells in primary culture (fig. 1a, inset). Stimulation of primary stellate cell contractile force by endothelin-1 was dose dependent and saturable (fig. 1a). Contractile force generation by primary stellate cells began to reach a plateau of 128 ± 17 dyn at ~2 n*M* endothelin-1. The effects of endothelin-1 on the contraction of immortalized stellate cells were also investigated. Endothelin-1 triggered a rapid and substantial augmentation of contractile force by immortalized stellate cells (fig. 1b, inset). Contractile force generation by immortalized stellate cells was also saturable and dependent on the endothelin-1 dose (fig. 1b). Endothelin-1-stimulated contractile force generation achieved a plateau of 114 ± 20 dyn at ~2 n*M* endothelin-1 in immortalized stellate cells.

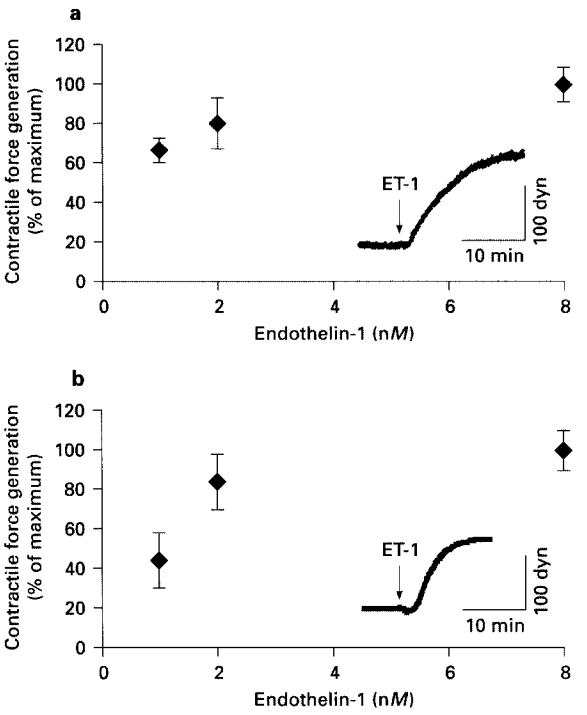

Fig. 1. Endothelin-1 stimulates contractile force generation by primary and immortalized stellate cells. **a** Endothelin-1 dose response relationship for contractile force generation by stellate cells in primary culture. Data are presented as mean contractile force generation as a percentage of maximal force ± SEM. Each point represents at least three independent experiments. Inset: representative contractile force tracing from primary stellate cells stimulated with 2 n*M* endothelin-1 (ET-1) at the time indicated. **b** Endothelin-1 dose-response relationship for contractile force generation by immortalized stellate cells. Data are presented as mean contractile force generation as a percentage of maximal force ± SEM. Each point represents at least three independent experiments. Inset: representative contractile force tracing from immortalized stellate cells stimulated with 2 n*M* endothelin-1 (ET-1) at the time indicated.

Endothelin-1 Stimulates Myosin Activation in Stellate Cells

If myosin mediates stellate cell contraction in response to endothelin-1, then endothelin-1 should stimulate the activation of myosin. Myosin activation was studied by measuring the phosphorylation of myosin and by examining the organization of the actin-myosin cytoskeleton. Treatment of immortalized stellate cells with endothelin-1 enhanced myosin phosphorylation in a dose-dependent and saturable manner (fig. 2a, b). Endothelin-1 treatment of immortalized stellate cells also enhanced actin polymerization and stress fiber formation (fig. 2c–f). As evidenced by punctate labeling along the axis of the stress fibers, endothelin-1 additionally stimulated co-localization of myosin, initially present diffusely throughout the cytoplasm, with polymerized actin (fig. 2c–f).

Antagonism of Myosin Activation Blocks Contractile Force Generation by Stellate Cells

To further dissect the role of myosin in endothelin-1-stimulated stellate cell contractile force generation, we used the selective endothelin-A receptor antagonist BQ-123. First, we demonstrated that BQ-123 completely abrogated endothelin-1-stimulated phosphorylation of myosin in immortalized stellate cells (fig. 3a). Then we showed that this endothelin receptor antagonist strongly blocked the generation of contractile force by these cells in response to endothelin-1 (fig. 3b). As a control experiment, we found that sarafotoxin, a selective endothelin-B receptor agonist, neither stimulated myosin phosphorylation (fig. 4a) nor contractile force generation (data not shown) by immortalized stellate cells.

The observation that inhibition of myosin activation blocked contractile force generation was confirmed with Y-27632, a selective inhibitor of rho-associated kinase [20]. Since rho-associated kinase causes a net increase in myosin phosphorylation, treatment with Y-27632 should result in a decrement in the phosphorylation of myosin. As predicted, Y-27632 led to a dose-dependent reduction in endothelin-1-stimulated myosin phosphorylation in

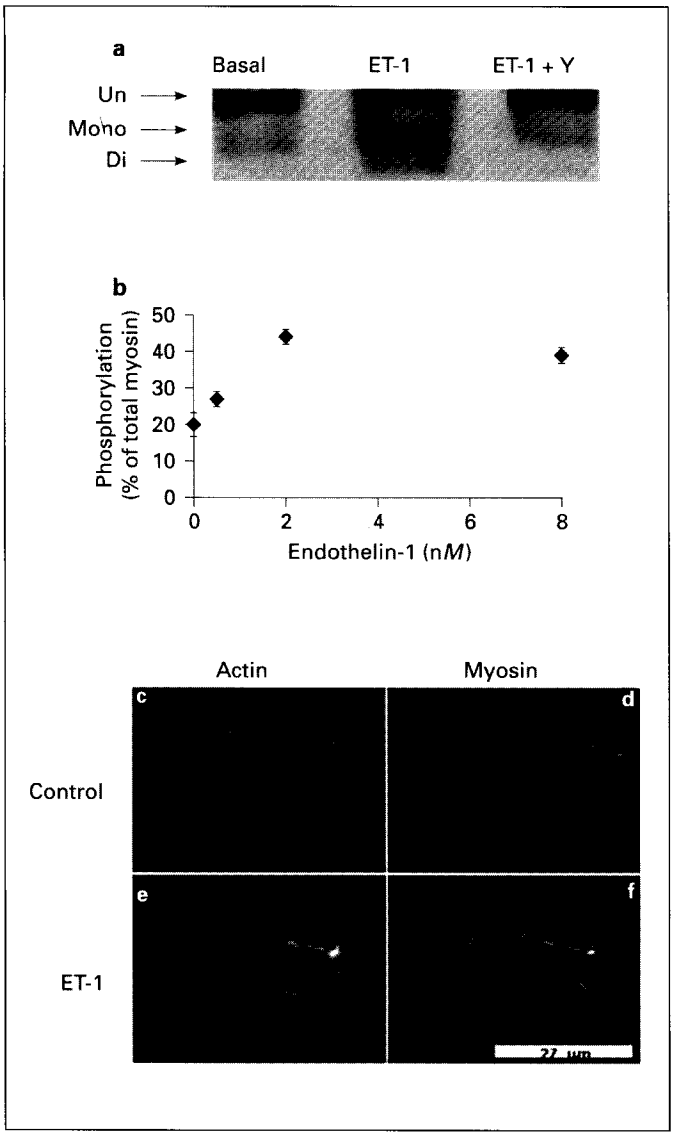

Fig. 2. Endothelin-1 induces myosin activation in immortalized stellate cells. **a** Representative autoluminogram showing unphosphorylated (Un), monophosphorylated (Mono), and diphosphorylated (Di) forms of myosin in cells treated with carrier, 8 n*M* endothelin-1 (ET-1), and 8 n*M* endothelin-1 with 10 μ*M* Y-27632 (ET-1 + Y). **b** Endothelin-1 dose-response relationship for myosin phosphorylation. Phosphorylation is presented as the amount of phosphorylated myosin species as a percentage of total myosin. Data are presented as means ± SEM. Each point represents at least three independent experiments. **c–f** Representative fluorescent images of an untreated (**c**, **e**) and an 8 n*M* endothelin-1 (**d**, **f**)-treated stellate cell. **c**, **d** Cells stained for polymerized actin with phalloidin. **e**, **f** Cells stained with an antibody directed against myosin.

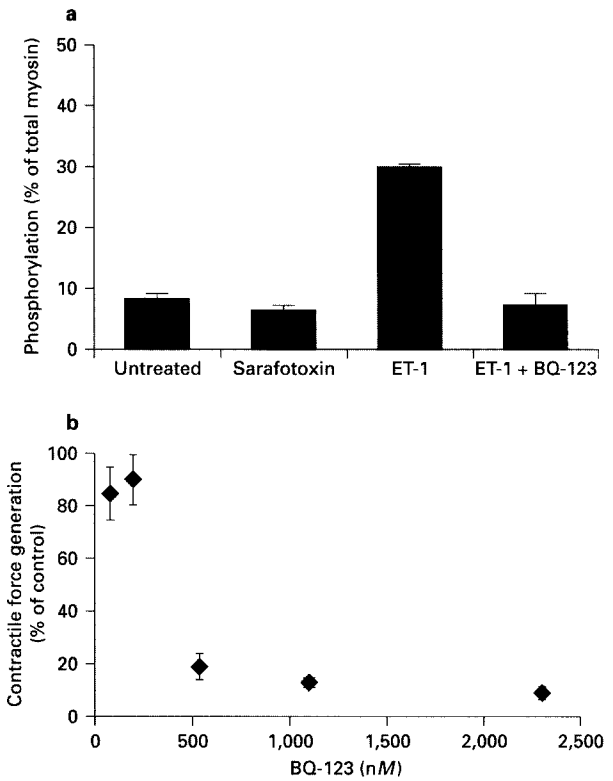

Fig. 3. Effects of endothelin receptor agonists and antagonists on myosin phosphorylation and contractile force generation by immortalized stellate cells. **a** The effects of no treatment (untreated), 100 n*M* sarafotoxin, 8 n*M* endothelin-1 (ET-1), and 8 n*M* endothelin-1 with 1 μ*M* BQ-123 (ET-1 + BQ-123) on myosin phosphorylation. Phosphorylation is presented as the amount of phosphorylated myosin species as a percentage of total myosin. Data are presented as means ± SEM. Each bar represents at least three independent experiments. **b** BQ-123 dose-response relationship for endothelin-1 (8 n*M*)-stimulated contractile force generation. Contractile force generation presented as a percentage of the contractile force generated by 8 n*M* endothelin-1 in the absence of BQ-123 (control). Data are presented as means ± SEM. Each point represents at least three independent experiments.

immortalized stellate cells (fig. 2a, 4a). Y-27632 also inhibited endothelin-1-stimulated contractile force generation with a similar dose dependence (fig. 4b).

Discussion

Although stellate cell contraction in response to endothelin-1 is believed to play an important role in the regulation of sinusoidal blood flow and the development of

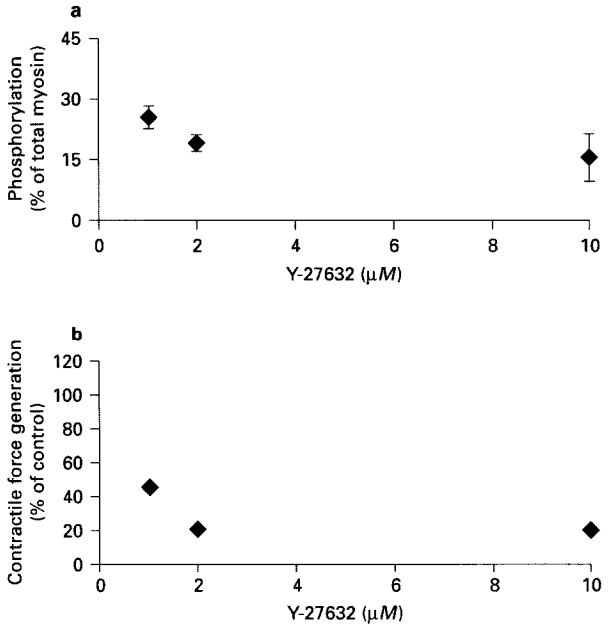

Fig. 4. Y-27632 inhibits myosin phosphorylation and contractile force generation by immortalized stellate cells. **a** Y-27632 dose-response relationship for endothelin-1 (8 n*M*)-stimulated myosin phosphorylation. Phosphorylation is presented as the amount of phosphorylated myosin species as a percentage of total myosin. Data are presented as means ± SEM. Each point represents at least three independent experiments. **b** Y-27632 dose-response relationship for endothelin-1 (8 n*M*)-stimulated contractile force generation. Contractile force generation presented as a percentage of the contractile force generated by 8 n*M* endothelin-1 in the absence of Y-27632 (control). Each point represents the mean of at least two experiments.

hepatic fibrosis [4, 11, 13], the molecular mechanisms linking endothelin-1 to contractile force generation in this cell type have not been well characterized. In this study, we provide novel data suggesting that myosin mediates contractile force generation by stellate cells in response to endothelin-1.

Employing a method for directly and quantitatively measuring the force generated by contracting stellate cells recently established in this laboratory [18], we have demonstrated that endothelin-1 is a potent (i.e. half-maximal force induced at ~ 1 n*M* endothelin-1) stimulus for contractile force generation by stellate cells in primary culture. In addition, the contractile response of primary stellate cells to endothelin-1 was rapid and substantial. To test the role that myosin plays in endothelin-1-stimulated contractile force generation by stellate cells, we used a well-characterized SV40-immortalized rat stellate cell line [2, 8, 9, 20]. This immortalized stellate cell line has previously been used successfully as a model for stellate cells since they share many biochemical and physiological features with rat stellate cells in primary culture. The suitability of using this immortalized stellate cell line to test our hypothesis is supported by our findings that the contractile responses of these cells to endothelin-1 were very similar to those of primary stellate cells. Specifically, endothelin-1 stimulated contractile force generation with a potency in immortalized stellate cells (i.e. half-maximal force induced at ~ 1 n*M* endothelin-1) which was close to that observed in stellate cells in primary culture. Moreover, the rate and magnitude of the increase in contractile force generated by immortalized stellate cells in response to endothelin-1 was comparable to that measured in primary stellate cells.

This study provides several lines of evidence to support the hypothesis that myosin mediates contractile force generation by stellate cells in response to endothelin-1. Firstly, endothelin-1 stimulated myosin phosphorylation and contractile force generation with a similar dose dependence. Secondly, a concentration of endothelin-1 that stimulated contractile force generation also induced actin stress fiber assembly and co-localization of myosin to stress fibers. Thirdly, a concentration of the endothelin-A receptor antagonist BQ-123 that inhibited myosin phosphorylation also blocked contractile force generation. Finally, the rho-associated kinase antagonist Y-27632 inhibited myosin phosphorylation and contractile force generation with the same dose dependence. Y-27632 is a highly specific inhibitor of rho-associated kinase [20], a kinase that acts principally by inactivating myosin phosphatase, which dephosphorylates myosin [16]. Together these data suggest that endothelin-1 stimulates contractile force generation by stellate cells through the activation of myosin.

Based on the results of this study, we propose a model in which endothelin-1, released in response to hepatic injury by stellate cells, endothelial cells, and Kupffer cells [1, 5, 12], binds to endothelin-A receptors [5, 12, 25] causing enhanced myosin phosphorylation through G-protein-coupled activation of rho-associated kinase [16]. Phosphorylation of MRLC activates myosin, which co-localizes to bundles of polymerized actin, resulting in generation of contractile force by stellate cells. Accordingly, our data indicate that myosin mediates stellate cell contraction, and may consequently contribute to the regulation of sinusoidal resistance and hepatic fibrosis.

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