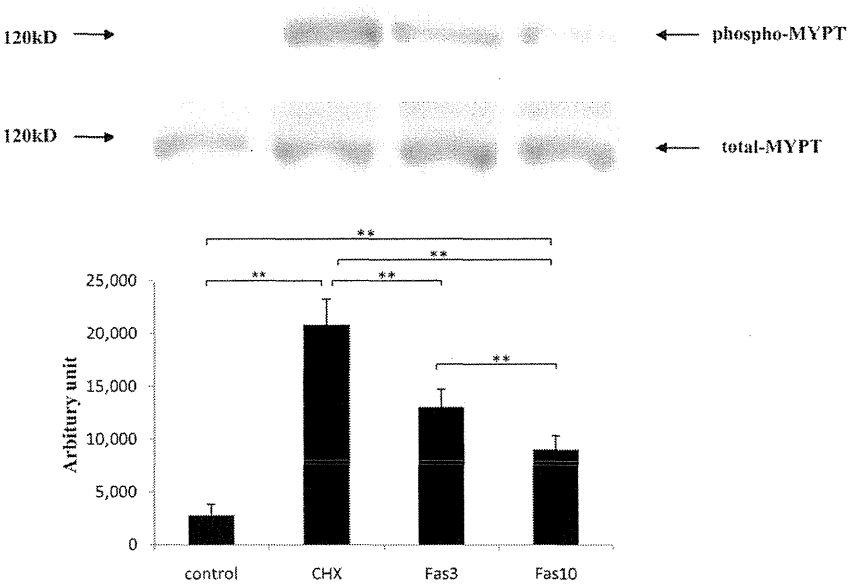

Fig. 5. Rho-kinase activity in the peritoneal tissues. The activity of Rho-kinase was evaluated by immunoblotting using antibody against phosphorylated MYPT, a substrate of Rho-kinase in control rats (control), rats with CHX-induced peritoneal damage (CHX) and rats with peritoneal damage treated with 3 mg/kg (Fas3) and 10 mg/kg (Fas10) of fasudil (upper panel). Immunoblots for total MYPT were loading controls (upper panel). Densitometric analysis was shown in the lower panel. **P < 0.01 between the two group, $n = 6$. The blots shown here were representative of three independent experiments.

changes, invasion of macrophages in the peritoneum was examined by immunostaining with CD68, a cell surface marker of macrophages. As shown in Figure 4A, the staining with CD68 was enhanced in the peritoneum of rats in the CHX group, which was significantly attenuated by treatment with fasudil in a dose-dependent manner (Fas3: 79.2% inhibition, P < 0.01, $n = 6$; Fas10: 88.4% inhibition, P < 0.01, $n = 6$). Immunostaining for α-SMA, one of the markers of fibrosis in the tissues was also examined. Staining was increased in the CHX group compared to the control group, which was also attenuated by treatment with fasudil in a dose-dependent manner (Fas3: 48.5 % inhibition, P = 0.51, $n = 6$; Fas10: 99.9% inhibition, P < 0.01, $n = 6$, Figure 4B, upper panel). Consistently, mRNA expression of α-SMA in the peritoneum of rats in the CHX group was enhanced in comparison with that in control rats (P < 0.01, $n = 6$, Figure 4B, lower panel), which was significantly attenuated by treatment with fasudil (Fas3: 46.7% inhibition, P = 0.54, $n = 6$; Fas10: 99.9% inhibition, P < 0.01, $n = 6$, Figure 4B, lower panel).

Rho-kinase activity in the peritoneal tissues

In order to explore the role of Rho-kinase in the pathogenesis of CHX-induced peritoneal fibrosis, the activity of Rho-kinase was evaluated by immunoblotting using an antibody against phosphorylated MYPT, a substrate of Rho-kinase. Rho-kinase activity in the peritoneum of rats in the CHX group was enhanced when compared with that of control rats (Figure 5, 3.5-fold induction versus control group, P < 0.01, $n = 6$). The activation of Rho-kinase was inhibited by treatment with fasudil (Figure 5, Fas3 group: 43.6% inhibition, P < 0.01 versus CHX group, $n = 6$; Fas10 group: 74.3% inhibition, P < 0.01 versus CHX group, $n = 6$). These data indicated that Rho/Rho-kinase pathway was activated in CHX-induced peritoneal damage, which was successfully inhibited by the treatment with fasudil.

Effects of Y-27632 on TGF-β expression and VEGF production in MeT-5A cells

To examine whether the effects of Rho-kinase inhibition was its direct action on mesothelial cells, an *in vitro* study utilizing the mesothelial cell line, MeT-5A cells, was performed. Stimulation of MeT-5A cells with high glucose (200 mmol/L) upregulated mRNA expression of TGF-β 10.5-fold as compared with that of quiescent cells (P < 0.01), which was inhibited by pretreatment with the Rho-kinase inhibitor, Y-27632, in a dose-dependent manner (Figure 6A, 5 μmol/L, Y5: 69.3% inhibition, P < 0.05 $n = 5$; 10 μmol/L, Y10: 81.5% inhibition, P < 0.01 versus glucose-stimulated cells, $n = 5$). Secretion of VEGF in the medium was increased in glucose-stimulated MeT-5A cells (3.0-fold induction, P < 0.05 versus quiescent cells, $n = 5$), which was also inhibited by Y-27632 in a dose-dependent manner (Figure 6B, 5 μmol/L, Y5: 14.7% inhibition, P < 0.05 $n = 5$; 10 μmol/L, Y10: 25.2% inhibition, P < 0.05 versus glucose-stimulated cells, $n = 5$). These data implied that the effects of Rho-kinase inhibition were partly due to direct action on mesothelial cells.

Discussion

During long-term PD, morphological and functional changes of the peritoneal membrane lead to ultrafiltration failure and inefficient elimination of solutes. Typical

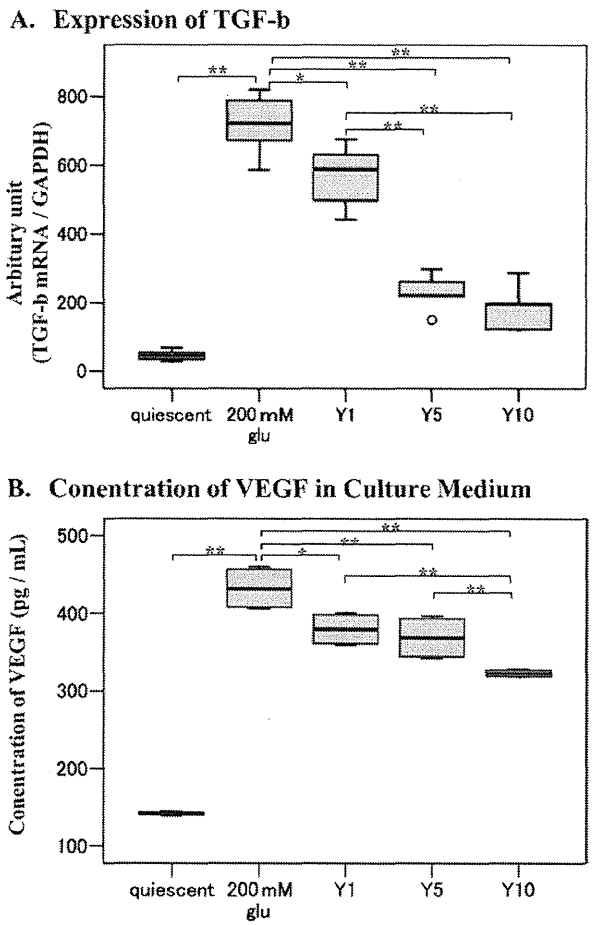

Fig. 6. Effects of Y-27632 on TGF-β expression and VEGF production in MeT-5A cells. Serum-starved mesothelial cells (quiescent) were stimulated with high glucose (200 mmol/L glucose) in the presence or absence of Rho-kinase inhibitor, Y-27632 at concentrations of 1 μmol/L (Y1), 5 μmol/L (Y5) and 10 μmol/L (Y10). The mRNA expression of TGF-β in the cell (upper panel) and the concentration of VEGF in the medium (lower panel) were analyzed by real-time PCR and enzyme-linked immunosorbent assay, respectively. **P < 0.01 versus CHX group, *P < 0.05 versus CHX group, $n = 5$.

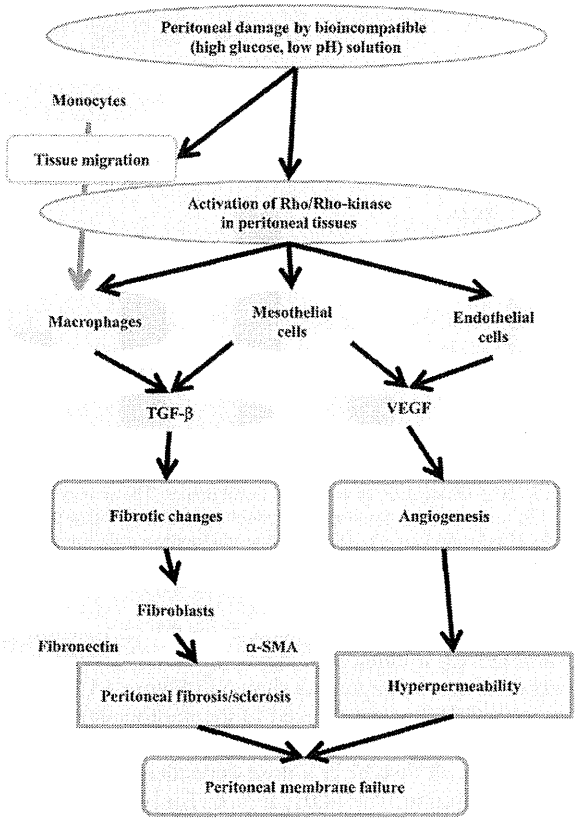

Fig. 7. Schema depicting the molecular mechanism for peritoneal damage through the activation of Rho/Rho-kinase pathway. Peritoneal tissue damage is induced by bioincompatible dialysis solution, which provoked migration of inflammatory cells and activation of the Rho/Rho-kinase pathway in each cell type present in the peritoneal tissues, monocytes/macrophages, mesothelial cells and endothelial cells. Activation of the Rho/Rho-kinase pathway in the peritoneal tissues induced tissue fibrosis and angiogenesis through the increased expression of TGF-β and VEGF, respectively. Angiogenesis leads to hyperpermeable peritoneal tissues. Both fibrosis and angiogenesis deteriorate peritoneal membrane function and result in ultrafiltration failure or increased transport of small solutes. Blocking this pathway halts these tissue alterations and prevents peritoneal membrane failure.

morphological alterations of the peritoneal membrane are submesothelial fibrosis, angiogenesis and inflammatory cell infiltration [29]. These peritoneal changes of PF and PS result in a serious clinical complication, EPS, although other factors than PS were involved in its pathogenesis (Figure 7). In the present study, we utilized a rat model with CHX-induced PF, which has been reported to mimic the peritoneal pathological changes in long-term PD patients, such as the pathological changes observed in PS and EPS [30, 31]. We demonstrated that the Rho-kinase inhibitor, fasudil successfully prevented the progression of PS. In addition, as shown in the present study (Figure 1), this model has a macroscopic appearance of EPS in the peritoneum and small intestine. Using this model, several reagents have been tested as therapeutic strategies against PS, including rennin–angiotensin system blockade [32], erythropoietin [24] and antifibrotic agent, pirfenidone [33]. In this study, we demonstrated that administration of fasudil inhibited the peritoneal fibrotic changes and blocked progression to EPS, which can constitute a novel therapeutic strategy for the prevention of EPS in long-term PD patients.

One of the first steps of PF is inflammatory damage to the mesothelial tissues, which is initiated by the recruitment of inflammatory cells. Another mechanism is that glucose degradation products, directly through the action receptor for advanced glycation end-products, could upregulate TGF-β and cause fibrosis. The damage of peritoneal mesothelial cells cause the peritoneal adhesion and dysfunction. Histological findings of damaged peritoneum in our rat models revealed that macrophages (CD68-positive cells) migrating from vascular tissues move on the surface of the peritoneum and reside in a line (Figure 4A). Studies in vascular tissues have suggested that monocyte recruitment into the vessel wall is usually promoted by chemokines, such as monocyte chemoattractant protein-1, which is an early step in the process of arteriosclerosis [13]. Rho-kinase is involved in macrophage-mediated

formation of coronary vascular lesions in an *in vivo* porcine model and a Rho-kinase inhibitor, hydroxyl-fasudil markedly inhibited macrophage accumulation and migration [34]. In this study, as a result of the inhibitory effects of fasudil on macrophage migration, the initiation of peritoneal tissue inflammation was attenuated and the expressions of fibrotic factor, TGF-β, fibronectin and various inflammatory cytokines which, in part, were secreted by migrated inflammatory cells, were downregulated. These effects contributed to blocking the aggravation of peritoneal damages.

Various humoral factors from migrated macrophages or mesothelial cells were suggested to be involved in the development of PS. Among them, TGF-β plays the most essential role in the initiation of PS by EMT of peritoneal mesothelial cells [35, 36]. Consistently, blockade of TGF-β has been shown to prevent the development of PF in rat PF models [8]. Accumulating evidence has been reported that the Rho/Rho-kinase pathway participates importantly in the process of tissue fibrosis as well as EMT in several tissue culture systems [37–41]. We previously reported that Rho-kinase inhibitor attenuates renal inflammatory changes in remnant kidney models and that, through its inhibitory effects on the Rho/Rho-kinase pathway, the T-type calcium channel blocker attenuated renal fibrotic changes of the rat model [42]. In the present study, the Rho-kinase inhibitor downregulated the increased expression of TGF-β and fibronectin in the peritoneal membrane of rats with CHX-induced PF. Furthermore, fasudil markedly inhibited the expression of α-SMA mRNA and decreased α-SMA immunostaining, which suggested its inhibitory effects on the fibrotic changes by mesothelial cells. Finally, Masson's trichrome staining revealed that the Rho-kinase inhibitor reduced thickening of the peritoneal membrane and decreased the fibrotic component. The present study provides a novel therapeutic strategy for prevention against the initiation and the progression of PF and its serious complication, EPS. The mechanisms involved in this PF were summarized in Figure 7.

Besides inflammation and fibrosis, an increased number of capillaries are also related to peritoneal membrane failure [1, 43]. Angiogenesis is involved in elevation of small-solute transport across the peritoneal membrane and in ultrafiltration failure. Peritoneal expression of VEGF is correlated with the degree of angiogenesis [44]. Our rat models of peritoneal damage presented with the increased number of capillaries and the increased expression of VEGF in the peritoneal membrane, which were attenuated by treatment with fasudil. Y-27632 inhibited secretion of VEGF from MeT-5A cells, although the expression levels in the cells were not altered (data not shown). Therefore, inhibitory effects by fasudil resulted from its effects on endothelial cells or on transdifferentiated mesothelial cells because transformed not intact mesothelial cells are an important source of VEGF in PD patients [45]. The expression of VEGF was regulated by the Rho/Rho-kinase pathway in vascular endothelial cells [46]. Our *in vitro* data indicated that, in addition to endothelial cells, peritoneal mesothelial cells also produced VEGF, which might contribute to angiogenesis in the submesothelial cell layer in the peritoneum and to the regulation of peritoneal permeability. Y-27632 blocked secretion of VEGF from MeT-5A cells, which underscored activation of the Rho/Rho-kinase pathway as a potential mechanism for angiogenesis in the damaged peritoneum. Bleeding through the neovascular tissues created an accumulated fibrin layer and resulted in the cocoon formation. As evident in the improvement in macroscopic findings of peritoneum in rats treated with fasudil, inhibition by Rho-kinase prevented bleeding and further damage to peritoneal tissues. These mechanisms involved in angiogenesis were summarized in Figure 7.

In the present study, peritoneal sclerosing models were made by injection of CHX solution intraperitoneally. This model does not precisely reflect the pathology of EPS and PS in PD patients. We were also unable to consider the extent to which uremia affected peritoneal membrane damages. Moreover, we observed that since peritoneal damages were induced by the intraperitoneal injection of CHX in this model, the affected lesion appeared to be widely dispersed, which caused the values of CHX group with wide variances. However, several studies already have shown similarities of pathological changes in peritoneal tissues between this models and long-term PD patients [24, 30, 31]. In addition, in an *in vitro* study, we utilized MeT-5A cell line whose phenotype is not completely similar to that of peritoneal mesothelial cells [26]. In spite of these limitations, this study demonstrated that the Rho-kinase inhibitor ameliorated the development of peritoneal damage. Since no promising therapeutics have been established for peritoneal injury in PD patients, the Rho-kinase inhibitor may be a novel strategy as a protective agent for peritoneal damages and the ensuing PS.

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Conflict of interest statement. None declared.

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