Endothelin-1 Suppresses Plasma Membrane Ca^{++} -ATPase, Concomitant with Contraction of Hepatic Sinusoidal Endothelial Fenestrae

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Intracytoplasmic free calcium ions (Ca^{++}) are maintained at a very low concentration in mammalian tissue by extruding Ca^{++} from the cytoplasm against a steep extracellular Ca^{++} concentration gradient, mainly through the activity of plasma membrane Ca^{++} pump-ATPase. The present study aimed to elucidate how endothelin-1 (ET-1) affects the morphology of sinusoidal endothelial fenestrae and ultrastructural distribution of plasma membrane ATPases and intracytoplasmic free Ca^{++} in isolated rat hepatic sinusoidal endothelial cells. Sinusoidal endothelial fenestrae were observed by scanning electron microscope. Ando's electron cytochemical method was used for ultrastructural localization of Ca^{++} - Mg^{++} -ATPase activity, electron immunogold postembedding method for Ca^{++} pump-ATPase immunoactivity, and antimonate method for intracytoplasmic free Ca^{++} . Addition of ET-1 to sinusoidal endothelial cells significantly decreased Ca^{++} - Mg^{++} -ATPase activity and Ca^{++} pump-ATPase expression and increased intracytoplasmic free Ca^{++} concentration, concomitant with a decrease in diameter of sinusoidal endothelial fenestrae. Co-treatment with Bosentan abolished the actions of ET-1. These results suggest that ET-1 suppresses Ca^{++} - Mg^{++} -ATPase activity and Ca^{++} pump-ATPase expression on the plasma membrane of sinusoidal endothelial fenestrae, thereby attenuating the extrusion of intracytoplasmic free Ca^{++} into the extracellular space, leading to an increased concentration of intracytoplasmic free calcium ions and contraction of sinusoidal endothelial fenestrae. (*Am J Pathol* 2003, 162:557–566)

Hepatic sinusoidal endothelial cells (SECs) possess fenestrae mostly arranged in sieve plate-like pores. These fenestrae lack a diaphragm and a basal lamina and therefore are an open connection between the lumen of the sinusoid and the space of Disse.¹ They are not single and independent pores but are interconnected labyrinth-like structures.² Actin microfilaments and calmodulin are closely associated with the endothelial plasma membrane, suggesting that in the presence of calcium ions, calmodulin and actin microfilaments may be involved in the contraction and dilatation of endothelial cell fenestrae.^{2–4}

The Ca^{++} pump of the plasma membrane extrudes Ca^{++} from the cytoplasm against a steep extracellular concentration gradient, and is essential for homeostatic maintenance of a low concentration of intracellular Ca^{++} .⁵ By immunofluorescence and immunoelectron microscopic study, the Ca^{++} pump in capillary endothelial cells and visceral smooth muscle cells is found to be 18- to 25-fold more concentrated in the invaginated caveolar membrane compared with the noncaveolar portion of the plasma membrane.^{6,7} Our recently study demonstrated localization of calcium pump-dependent adenosine triphosphatase (Ca^{++} pump-ATPase) in hepatic sinusoidal endothelial fenestrae (SEF).⁸

Endothelins (ETs) are a family of three potent vasoconstrictor peptides. ET-1 was first identified in the supernatant of cultured endothelial cells.⁹ Two other forms of ET have subsequently been characterized and designated ET-2 and ET-3, which differ from ET-1 by two and six amino acids, respectively.^{10,11} Recently the authors have reported that ET-1 induces contractions of the SEF not only *in vivo*¹² but also *in vitro*.¹³ The present study was designed to elucidate the subcellular mechanism of SEF contraction by focusing on the electron cytochemical distribution of plasma membrane Ca^{++} - Mg^{++} -ATPase, Ca^{++} pump-ATPase, and intracytoplasmic free calcium ions in SECs.

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Materials and Methods

Experimental Animals

Male Wistar strain rats weighing 150 to 180 g were used in this study. Animals were housed in individual cages and allowed free access to chow and water until the start of the study. All animal procedures in the present experiments were performed in compliance with the standard guidelines for experiments of the School of Medicine, Keio University. In this study, we investigated 10 animals.

Isolation, Purification, and Culture of SECs

SECs were isolated by a modified method of Braet and colleagues.¹⁴ In brief, the liver of a male Wistar rat was perfused with Ca^{++} - Mg^{++} -free Hanks' balanced salt solution followed by 0.6% collagenase A (Sigma type 1) via a polyethylene catheter inserted into the portal vein trunk. After incubation of the fragmented tissue in the same solution, the resulting cell suspension was centrifuged at $100 \times g$ for 10 minutes to remove the parenchymal cells. The supernatant containing a mixture of sinusoidal liver cells was then layered on top of a two-step Percoll gradient (25 to 50%) and centrifuged for 20 minutes at $900 \times g$. The intermediate zone located between the two density layers was enriched in SECs. The purity of SECs was further increased by selective adherence of Kupffer cells and spreading of the SECs on collagen. SECs were seeded on coated coverglass (Iwaki, Tokyo, Japan) placed in six-well (Nalge Hune International (Naperville, IL) or two-well dishes (Falcon 3001). Serum-free SEC culture medium was RPMI 1640 medium supplemented with 2 mmol/L of L-glutamine and 100 $\mu\text{g}/\text{ml}$ of gentamicin. The dishes were incubated in a CO_2 gas incubator at 37°C for 24 hours.

Treatment of Cultured Cells with ET-1

Twelve-hour cultures of hepatic SECs were washed with L-15 culture medium (37°C) and divided into three groups. The control group received no treatment. The ET-1-treated group was incubated with 1 nmol/L of ET-1 (Sigma Chemical Co., St. Louis, MO) for 30 minutes. The ET-1 and Bosentan-treated group was pretreated with 10 $\mu\text{mol}/\text{L}$ of Bosentan (a gift from Roche Chemical Co.) for 20 minutes and then treated with ET-1 for 30 minutes. We investigated 20 dishes for each group.

The concentrations of ET-1 and Bosentan used in the experiments were determined in preliminary experiments. We compared four concentrations of ET-1 (10^{-7} , 10^{-8} , 10^{-9} , and 10^{-11} mol/L) for the contraction of SEF. Although both 10^{-8} and 10^{-9} mol/L effectively caused contraction, we chose the lower concentration of 1 nmol/L for subsequent experiments. A previous study reported that Bosentan blocked all nonpeptide ET receptors at a concentration of 10^{-5} mol/L,¹⁵ we tested three concentrations of Bosentan (10^{-4} , 10^{-5} , 10^{-6} mol/L) to determine the optimal concentration for use in our experimental system. Although 10^{-4} mol/L blocked both ETAR and

ETBR more completely than 10^{-5} mol/L, we chose the lower concentration of 10^{-5} mol/L in this study.

Electron Cytochemical Detection of Plasma Membrane Ca^{++} - Mg^{++} -ATPase in Isolated SECs

The SECs cultured on a coverglass were fixed in a mixture of 1% paraformaldehyde and 0.5% glutaraldehyde in 0.1% cacodylate buffer (pH 7.2) for 10 minutes at 4°C , and were then washed overnight with 0.25 mol/L of sucrose in 0.1% cacodylate buffer. The SECs on coverglass were incubated for 30 minutes at 37°C in a reaction medium containing 250 mmol/L of glycine buffer (pH 9.0), 3 mmol/L of ATP (Sigma Chemical Co.) as the substrate, 10 mmol/L of CaCl_2 as the activator, 2.5 mmol/L of leuamisole (KW-2-LE-T; Kyowa, Tokyo, Japan), and 2 mmol/L of lead citrate as the capture reagent. The cells were then rinsed in 0.1 mol/L of cacodylate buffer (pH 7.2) containing 0.25 mol/L of sucrose, postfixated with 1% osmic acid for 30 to 60 minutes at 4°C ,¹⁶ and dehydrated through a graded series of ethanol solutions. The coverglass was placed with the SECs on the upper surface. Then a plastic capsule containing melted epoxy resin was placed on the cells. After polymerization of the resin, the coverglass was removed from the resin by heating. The embedded cells were cut with an LKB ultratome, poststained with uranyl acetate, and examined under a JEM 1200 EX electron microscope at an accelerating voltage of 80 kV. The electron-dense reaction products detected on the endothelial fenestral membranes show the activity of Ca^{++} -ATPase.

Immunoelectron Microscopy for Ca^{++} Pump-ATPase in Isolated SECs

According to a modification of Berryman and Rodewald^{17,18}, some samples of isolated cultured cells were fixed in periodate-lysine-paraformaldehyde for 1 hour. After washing the cells with several changes of cold 0.1-mol/L phosphate buffer containing 3.5% sucrose and 0.5-mmol/L calcium chloride for 2 hours, free aldehyde was quenched in sucrose-phosphate buffer containing 50 mmol/L of ammonium chloride and 0.5 mmol/L of calcium chloride (pH 7.4) for 1 hour at 0°C . To remove the phosphate ions, the cells were rinsed four times with cold 0.1-mol/L maleate buffer (final pH 6.0) containing 3.5% sucrose, pH 6.5 (four times for 15 minutes each), and was then poststained for 10 minutes at 0°C with 2% uranyl acetate in sucrose-maleate buffer (final pH 6.0). Distilled water (final pH 4.0), 0.05 mol/L maleate buffer (final pH 4.2), and veronal acetate buffer (final pH 4.2) were also used as vehicles to prepare 2% uranyl acetate for poststaining for 5 minutes at 0°C . The cells were dehydrated in 50, 70, 90, and 100% acetone sequentially, at -20°C for 45 minutes each, and were then embedded at -20°C in LR Gold (London Resin Co., Hants, UK). To block nonspecific binding sites, the grids were placed on drops

Figure 1. Electron cytochemical reaction of Ca^{++} - Mg^{++} -ATPase activity in hepatic SECs. **a:** Ultrastructural localization of Ca^{++} - Mg^{++} -ATPase activity in the untreated control SECs. Cytochemical reaction products of Ca^{++} - Mg^{++} -ATPase activity are localized on the outer surface of the plasma membrane of the labyrinth-like SEF. **b:** Ultrastructural localization of Ca^{++} - Mg^{++} -ATPase in SECs treated with ET-1. Marked decrease in cytochemical reaction products of Ca^{++} - Mg^{++} -ATPase activity is observed on the outer surface of the plasma membranes of the labyrinth-like SEF, compared to control SECs. **c:** Ultrastructural localization of Ca^{++} - Mg^{++} -ATPase in SECs treated with ET-1 and Bosentan. No decrease in cytochemical reaction products of Ca^{++} - Mg^{++} -ATPase activity is observed compared to control SECs. **d:** Negative control (medium containing CaCl_2 , levamisole, lead citrate only, and no substrate). Counterstained with lead citrate. Scale bar, 1 μm . **Inset** shows higher magnification. C, SECs; f, SEF.

of 5% normal goat serum diluted in Tris-buffered saline. Then the grid was transferred successively on drops of primary antibody (Ca^{++} pump-ATPase; Sigma Biosciences, St. Louis, MO), secondary antibody, and 5-nm colloidal gold-conjugated anti-mouse IgG antibody (Cosmo Bio, Tokyo, Japan). Normal goat serum (5%)

diluted in Tris-buffered saline was used to dilute all antibodies and also for washing. After the final washing, the grids were fixed in glutaraldehyde for 5 minutes, rinsed in water, stained for 15 minutes in 2% aqueous osmium tetroxide, and then examined under a JEM 1200 EX electron microscope at an accelerating voltage of 80 kV.

Figure 2. Quantitative analysis of $\text{Ca}^{++}\text{-Mg}^{++}\text{-ATPase}$ in SEF using an image analyzer (high-speed color image analyzer SP500, Olympus). ET-1 significantly suppressed $\text{Ca}^{++}\text{-Mg}^{++}\text{-ATPase}$ on the plasma membrane of SEF, and this suppression is inhibited by Bosentan.

Western Blotting of Calcium Pump-ATPase in Isolated SECs

The SECs were lysed with a solution containing 200 mmol/L of octyl- α -D-glucopyranoside, 100 mmol/L of Tris-HCl, and 1 mmol/L of phenylmethyl sulfonyl fluoride for 1 hour at 4°C and centrifuged twice at 2000 rpm for 10 minutes each. The protein concentrations were determined by Bradford assay. Samples (30 μ g protein/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing condition on 7.5% acrylamide gel, and electrophoretically transferred to a nylon membrane (Hybond-N; Amersham). Filters were blocked with 1% dry milk and 1% bovine serum albumin in phosphate-buffered saline (PBS) with 0.02% Tween for 2 hours, probed with the anti- Ca^{++} pump-ATPase antibody (1:100) for 1 hour, rinsed in PBS, and then probed with an alkaline phosphatase-conjugated goat anti-mouse antibody (1:3000) for 30 minutes. After rinsing in PBS, color was developed with the Protoblot alkaline phosphatase detection system (Promega).

Potassium-Containing Antimonate Method for $[\text{Ca}^{++}]_i$ in Isolated SECs

SECs cultured on a glass coverslip were fixed with 1.2% glutaraldehyde buffered with 0.1 mol/L of phosphate buffer (pH 7.6) containing 2% potassium antimonate (Wako Chemicals, Osaka, Japan) for 1 hour at 4°C. After rinsing with 0.1 mol/L of phosphate buffer (pH 7.6) containing 0.25 mol/L of sucrose and 2.5% potassium antimonate, the cells were postfixed with 1.5% osmic acid in 0.03 mol/L of potassium phosphate buffer (pH 7.8) containing 1.5% antimonate for 60 minutes at 4°C. The cells were then dehydrated through a graded series of ethanol

solutions and embedded in Epon medium.¹⁹ These embedded cells were cut with an LKB ultratome, post-stained with uranyl acetate, and examined under a JEM 1200 EX electron microscope at an accelerating voltage of 80 kV.

Scanning Electron Microscopy

SECs cultured on a glass coverslip were fixed in 1.2% glutaraldehyde buffered with 0.1 mol/L of cacodylate buffer (pH 7.4) for 1 hour at 4°C, and postfixed with 1% osmium tetroxide in cacodylate buffer (pH 7.4) for 1 hour at 4°C. After dehydration in a graded series of ethanol solutions, the cultured cells were dried in a critical point apparatus (HCR-2; Hitachi, Tokyo, Japan)⁴ and coated with gold in a Hitachi vacuum-coating unit. The cell surfaces were observed in a scanning electron microscopy (Hitachi) at 15-kV acceleration voltage.

Statistics

Morphometric Semiquantitative Analysis of Plasma Membrane $\text{Ca}^{++}\text{-Mg}^{++}\text{-ATPase}$

Plasma membrane $\text{Ca}^{++}\text{-Mg}^{++}\text{-ATPase}$ activity on SECs was quantitatively analyzed on electron micrographs at the same magnification. The total length (A) of SEF plasma membrane was traced and the total area (B) of the cytochemical reaction products of $\text{Ca}^{++}\text{-Mg}^{++}\text{-ATPase}$ activity, ie, electron dense globular deposits, on the SEF membrane was determined using an image analyzer (high-speed color image analyzer SP500; Olympus, Tokyo, Japan). $\text{Ca}^{++}\text{-Mg}^{++}\text{-ATPase}$ was expressed as a percentage of B/A. The statistical significance of the difference between the two groups was assessed with Student's *t*-test, and $P < 0.05$ was regarded as indicating a significant difference. Data were expressed as the mean $\pm$ SEM.

Ca^{++} Pump-ATPase

The immunogold labeling in ultrathin sections of SECs was quantitated using the National Institutes of Health Mac Measure program, version 1.41. The SEF and non-SEF plasma membranes were selected randomly, and the numbers of gold particles per unit length of membrane were counted. The statistical significance of the difference between the two groups was assessed with Student's *t*-test, and $P < 0.05$ was regarded as indicating a significant difference. Data are expressed as the mean $\pm$ SEM.

Potassium-Containing Antimonate

Intracellular antimonate deposits were quantitatively analyzed on electron micrographs at the same magnification. The intracellular antimonate deposits in SECs were determined using a three-dimensional image analyzer (Winroof; Mitani Co., Tokyo, Japan). The statistical significance of the difference between two groups was

Figure 3. Ultrastructural localization of Ca^{++} pump-ATPase in the SECs. **a:** In the control cell, gold particles showing the presence of Ca^{++} pump-ATPase are localized mainly on the inner sites of plasma membranes of labyrinth-like SEF. **b:** In the ET-1-treated cell, gold particles showing the expression of Ca^{++} pump-ATPase on the inner sites of plasma membranes of labyrinth-like SEF is decreased compared to control cell. **c:** In the ET-1 and Bosentan-treated cell, no decrease in Ca^{++} pump-ATPase expression is observed compared to control cell. **d:** In the negative control (secondary antibody only). Uranyl acetate stained. Scale bar, 200 nm. **Arrowhead,** presence of Ca^{++} pump-ATPase. **Inset** shows lower magnification. C, SECs; f, SEF.

assessed with Student's *t*-test, and $P < 0.05$ was regarded as indicating a significant difference. Data are expressed as the mean $\pm$ SEM.

Alterations of Diameter of SEF

Based on scanning electron micrographs randomly taken at a magnification of $\times 10,000$, the diameters of 600 fenestrae in 20 SECs were measured. Statistical analysis was performed with Mann-Whitney *U*-test. Data are expressed as the mean $\pm$ SEM.

Results

Electron Cytochemical Reaction of Plasma Membrane Ca^{++} -ATPase in SECs

We first investigated plasma membrane Ca^{++} -ATPase in isolated SECs by electron cytochemistry. In the control, the electron dense cytochemical reaction products showing the presence of Ca^{++} -ATPase activity were demonstrated mainly on the outer surface of the invaginated plasma membrane of the labyrinth-like SEF in the isolated

Figure 4. Morphometric analysis of immunogold labeling for plasma membrane Ca^{++} pump-ATPase in SEF analyzed by the NIH Mac Measure program, version 1.41. ET-1 suppressed the expression of Ca^{++} pump-ATPase in SEF, and the suppression was inhibited by Bosentan.

SECs. However, these reaction products were rarely seen on the outer cell membrane of the SECs (Figure 1a).

In contrast, in SECs treated with ET-1, reaction products of Ca^{++} -ATPase on the outer surface of the plasma membranes of the labyrinth-like SEF were markedly decreased compared with the control cells (Figure 1b). Such a decrease was not observed in SECs treated with ET-1 and Bosentan (Figure 1c). Morphometric analysis of plasma membrane Ca^{++} -ATPase showed that ET-1 significantly suppressed Ca^{++} -ATPase on the plasma membrane of SEF, and this suppression was inhibited by Bosentan (Figure 2).

Immunoelectron Microscopic Localization of Ca^{++} Pump-ATPase in SECs

Next, we investigated the effect of ET-1 on Ca^{++} pump-ATPase expression in SEF by immunoelectron microscopy. In the control, electron-dense immunogold particles showing the presence of Ca^{++} pump-ATPase were mainly localized on the inner sites of the plasma membranes of labyrinth-like SEF (Figure 3a).

In the SECs treated with ET-1 (Figure 3b), gold particles showing the presence of Ca^{++} pump-ATPase were decreased in SEF, compared to the control cells. In cells treated with ET-1 and Bosentan, no decrease in Ca^{++} pump-ATPase was observed compared to the control (Figure 3c). Morphometric analysis of Ca^{++} pump-ATPase showed that ET-1 significantly suppressed Ca^{++} pump-ATPase on the plasma membrane of SEF, and such a decrease was inhibited by Bosentan (Figure 4).

Western Blotting of Calcium Pump-ATPase in Isolated SECs

To confirm the immunoelectron microscopic results, we investigated Ca^{++} pump-ATPase protein expression by

Figure 5. Western blot analysis of expression of Ca^{++} pump-ATPase protein expression in SECs. Samples containing 30 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 to 20% gels) and analyzed by blotting. **Lane 1**, control; **lane 2**, ET-1 treated; **lane 3**, ET-1 and Bosentan treated. Ca^{++} pump-ATPase protein expression is high in control and ET-1 and Bosentan-treated cells, and low in ET-1-treated cells. Positions of molecular mass markers are shown (kd).

Western blotting. Samples containing 30 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 to 20% gels) and analyzed by Western blotting. Ca^{++} pump-ATPase protein expression was high in untreated controls and ET-1 plus Bosentan-treated cells but very low in ET-1-treated cells (Figure 5).

Ultrastructural Localization of $[\text{Ca}^{++}]_i$ by Potassium-Containing Antimonate

Next, we examined $[\text{Ca}^{++}]_i$ by potassium-containing antimonate. In the control SECs, electron-dense cytochemical reaction products indicating the presence of $[\text{Ca}^{++}]_i$ were located mainly along the cell membrane (Figure 6; a to c). In the ET-1-treated SECs, ultrastructural distribution of $[\text{Ca}^{++}]_i$ was strong in the cytoplasmic components, especially in the SEF, and was slightly increased along the cell membrane (Figure 6; d to f). In the ET-1 plus Bosentan-treated SECs, the ultrastructural distribution of $[\text{Ca}^{++}]_i$ was mainly along the cell membrane, similar to the control SECs (Figure 6; g to i). Morphometric analysis of $[\text{Ca}^{++}]_i$ showed a significant increase in $[\text{Ca}^{++}]_i$ in ET-1-treated SECs (Figure 7).

Scanning Electron Micrographs of SEF

Finally, we examined the effect of ET-1 on the morphology of SEF by scanning electron microscopic observations. In the control hepatic SECs, there were two types of fenestration: sieved plate-like small pores and relatively isolated scattered pores (Figure 8a). In the ET-1-treated SECs, the diameters of SEF were decreased compared to the control SECs (Figure 8b). In ET-1 plus Bosentan-treated SECs, no reduction in diameter of SEF was observed compared to the control cells (Figure 8c). Morphometric analysis showed that the diameter of SEF was significantly decreased by treatment with ET-1, and this reduction was inhibited by co-treatment with Bosentan (Figure 9 and Table 1).

Discussion

Vasoactive agents play important roles in the pathogenesis of chronic liver injury.²⁰ ET, a pleiotropic peptide with

Figure 6. Ultrastructure of potassium-containing antimonate in the SECs. **a–c:** In the control cell, electron-dense cytochemical reaction products indicating the presence of $[Ca^{++}]_i$ are localized mainly along the plasma membrane. **d–f:** In the ET-1-treated cell, numerous fine deposits of reaction products are distributed in the cytoplasmic region, and there is a slight increase along the plasma membrane compared to the control. Scale bar, 1 μ m. **g–i:** In the ET-1 and Bosentan-treated cell, numerous fine deposits are observed along the plasma membrane. **Top** (low magnification) and **middle** figures (high magnification, SEF portion): electron micrograph of potassium-containing antimonate showing the presence of $[Ca^{++}]_i$. **Bottom:** $[Ca^{++}]_i$ in SECs analyzed by three-dimensional image analyzer. Uranyl acetate stained. Scale bar, 1 μ m. C, SECs; f, SEF.

potent vasoconstrictive properties, not only plays a role in controlling local vascular tone,²¹ but also has broad effects on growth and development.²²

In cirrhosis, the total amount of ETs produced was greater in SECs than in hepatic stellate cells, although the relative increase in ET-1 expression was higher in hepatic stellate cells than in SECs.²³ In liver injury, fibrogenesis occurs after activation of hepatic stellate cells, and the activation cascade includes features of proliferation, fibrogenesis, up-regulation of smooth muscle actin, and enhanced contractility.²⁴ However, the effects of ETs on SECs has not yet been characterized.

The diameter and number of fenestrae in SECs are not static but dynamic,³ and can be influenced by various agents such as bethanechol,²⁵ ethyl alcohol,²⁶ serotonin,⁴ and extracellular matrix.²⁷ In the present study, we demonstrated contraction of SEF by treating isolated SECs with ET-1.

Figure 7. Morphometric analysis of $[Ca^{++}]_i$ in the SECs from images of three-dimensional image analyzer.

Figure 8. Scanning electron micrographs of SEF in monolayer of SECs. **a:** In the control cell, two types of SEF are observed; sieve plate-like small pores and relatively large scattered pores. **b:** In ET-1-treated SECs, diameter and number of SEF are decreased compared to control. **c:** In ET-1 and Bosentan-treated SECs, no change in SEF diameter is observed compared to control cell. Counterstained with lead citrate. Scale bar, 1 μ m.

The cytoskeleton is well known to consist of three major components; microfilaments, intermediate filaments, and microtubules. Actin and myosin have been demonstrated to be involved in the contraction and relaxation activities of the sinusoidal fenestrae.^{3,28} Morphological observations indicated that the fenestrae were surrounded by an actomyosin-calmodulin-based cytoskeleton.³ There is evidence that SECs possess the apparatus for contraction.^{29,30} Myosin and actin might be involved in fenestral contraction, because activation of myosin light chain kinase and protein kinase C occurs in response to serotonin.³¹ This contraction is associated with rapid influx of Ca^{++} that is dependent on extracellular Ca^{++} .⁴ Attenuation of fenestral plasma membrane Ca^{++} - Mg^{++} -ATPase activity and Ca^{++} pump-ATPase expression by

ET-1 would cause an elevation of intracellular Ca^{++} and possibly inhibit extrusion of free Ca^{++} , resulting in contraction of the SEF. In our potassium-antimonate study, ET-1 induced an increase in intracytoplasmic $[\text{Ca}^{++}]_i$ in SECs, whereas this increase in $[\text{Ca}^{++}]_i$ was markedly suppressed by pretreatment with Bosentan. Contraction of SEF involves the Ca^{++} -calmodulin-actomyosin system.⁵ The present study suggests that ET-1 may suppress both plasma membrane Ca^{++} - Mg^{++} -ATPase activity and Ca^{++} pump-ATPase expression in the SEF, leading to the contraction of SEF because of an increase of intracytoplasmic $[\text{Ca}^{++}]_i$.

Plasma membrane Ca^{++} - Mg^{++} -ATPase activity was evidently localized on the outer surface of the SEF membrane, whereas the Ca^{++} pump-ATPase expression was localized at the inner site of the SEF membrane. Many reports have documented that plasma membrane Ca^{++} pump-ATPase plays a crucial role in maintaining a very

Figure 9. Morphometric analysis of diameters of SEF on scanning electron micrographs. Diameter distribution of fenestrae in control, ET-1-treated and ET-1 and Bosentan-treated SECs. *N* = number counted.

Table 1. Comparison of SEF Diameter between Control, ET-1-Treated, and ET-1 plus Bosentan-Treated SECs

	Fenestrae diameter (nm)	$\pm$ SEM
Control	218	39
Endothelin-1	157*	57
Endothelin-1 plus Bosentan	217	40

Results are expressed as mean $\pm$ SEM. Data are obtained from four different experiments.

*Significant difference in diameter of fenestrae is found in ET-1-treated group versus control group and ET-1 plus Bosentan-treated group ($P < 0.0001$, one-way factorial ANOVA).

low concentration of intracellular free Ca^{++} against a high concentration of extracellular free Ca^{++} through extrusion of intracellular free calcium ions.⁵ We demonstrated that ET-1 suppressed cytochemical Ca^{++} - Mg^{++} -ATPase activity and immunoreactive Ca^{++} pump-ATPase expression on the plasma membrane. There is a possibility that the cytochemical Ca^{++} - Mg^{++} -ATPase activity may correspond to the Ca^{++} pump-ATPase activity related to active extrusion of Ca^{++} from the cytoplasm, because Ca^{++} pump-ATPase has a plasma membrane-penetrating type structure.³²

The SEF were found to be a labyrinth-like structure of the deeply invaginated plasma membrane. SEF are very similar to fused and interconnected racemose clusters of caveolae.³³ In fact, we have reported that the caveolin-1 was localized in some parts of the invaginated labyrinth-like structure of SEF.³⁴ Caveolae have been a focus of interest as signal-transducing subcompartments in the cell, and are putative sites of Ca^{++} influx.^{7,35} Caveolae may be the sites where inositol 1,4,5-triphosphate is generated. Furthermore, inositol 1,4,5-triphosphate receptor-like protein has been localized in plasmalemmal caveolae,³⁴ and caveolae contain Ca^{++} - Mg^{++} -ATPase activity.³⁶ The Ca^{++} - Mg^{++} -ATPase activity and inositol 1,4,5-triphosphate receptor-like protein co-localized in caveolae may work coordinately to sequester Ca^{++} from caveolar vesicles. We speculate that similar mechanisms may also occur in SEF.

The present results suggest that in conditions of increased ET-1 such as cirrhosis, the increased ET-1 may induce not only contraction of hepatic stellate cells but also reduction in diameter and number of SEF,³⁷ thus increasing sinusoidal vascular resistance and contributing to the pathogenesis of portal hypertension.²⁰

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