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Involvement of Protein Phosphatase-1 in Cytoskeletal Organization of Cultured Endothelial Cells

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Abstract The phosphorylation and dephosphorylation of cytoskeletal proteins regulate the shape of eukaryotic cells. To elucidate the role of serine/threonine protein phosphatases (PP) in this process, we studied the effects of calyculin A (CLA), a potent and specific inhibitor of protein phosphatases 1 (PP-1) and 2A (PP-2A) on the cytoskeletal structure of cultured human umbilical vein endothelial cells (HUVECs). The addition of CLA (5 min) caused marked alterations in cell morphology, such as cell constriction and bleb formation. Microtubules and F-actin were reorganized, becoming markedly condensed around the nucleus. Although the fluorescence intensity of phosphoamino acids was not significantly different according to immunocytochemistry between cells with and without CLA, polypeptides of 135, 140, 158, and 175 kDa were specifically phosphorylated on serine and/or threonine residues. There was no significant effect on tyrosine residues. The effects of CLA on cytoskeletal changes and protein phosphorylation were almost completely inhibited by the non-selective kinase inhibitor, K-252a. The effect of CLA on cell morphology was at least 100 times more potent than that of okadaic acid, consistent with the inhibitory potency against PP-1. The catalytic subunit of PP-1 was also identified in HUVECs by Western blotting with its monoclonal antibody. These results suggest that PP-1 is closely involved in sustaining the normal structure of the cytoskeleton.

Key words: calyculin A, protein phosphatase, cytoskeleton, endothelial cell, immunocytochemistry

Serine/threonine protein phosphatase 1 and 2A (PP-1/PP-2A) play important roles in various functions of eukaryotic cells [Chartier et al., 1991; Hirano et al., 1992; Kreienbuhl et al., 1992; Yano et al., 1994; Takai et al., 1987; Sacher et al., 1992]. We reported that okadaic acid (OA) and calyculin A (CLA), which are potent and cell-permeable inhibitors of PP-1/PP-2A, induced significant morphological changes not only in thrombin stimulated, but in resting platelets [Yano et al., 1994]. OA/CLA very rapidly induced the reorganization of microtubules and actin filaments, leading to a change in platelets from the smooth discoid type to the oval type with short pseudopods (Yano et al., unpublished data). Similar morphological effects of OA on fibroblasts [Hirano et al., 1992] and leukocytes [Downey et al., 1993] have been reported, but their effects on endothelial cells have never been studied. Furthermore, the involvement of protein phosphorylation in the cytoskeletal reorganization of endothelial cells is still poorly understood.

Protein phosphorylation mainly occurs on serine, threonine, and tyrosine residues. Although the correlation between tyrosine phosphorylation and cell structural changes in various cells have been investigated, the involvement of serine and/or threonine phosphorylation in the cytoskeletal structure of endothelial cells has not been studied. Here, we demonstrate that PP-1 plays a predominant role in sustaining the normal cell structure of human endothelial cells.

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MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated sources. Antibodies: Mouse anti-alpha tubulin monoclonal antibody (Cedarlane

Laboratories, Ltd., Ontario, Canada), mouse monoclonal antibodies against phosphoserine, phosphothreonine (Bio Makor, Rehevot, Israel), and anti-phosphotyrosine mouse monoclonal antibody (PY 20) (Leineo Technology, Inc., St. Louis, MO); fluorescein-conjugated goat anti-mouse IgG (Organon Teknika Co., Durham, NC); peroxidase conjugated goat anti-mouse IgG (Organon Teknika Co.); anti-protein phosphatase 1 rabbit monoclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY). Other reagents: Calyculin A and okadaic acid (Wako Pure Chemical Co., Osaka, Japan); rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR); PVDF protein sequencing membrane (Bio Rad, Laboratories, Richmond, CA); CHL-MCDB131 (Kurorera, Co., Tokyo, Japan).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained by incubating surgically resected human umbilical cord for 90 min at room temperature in medium MCDB 131 containing 1,000 pu/ml of dispase. Cells were grown at 37°C in tissue culture flasks in MCDB 131 media containing 10% fetal bovine serum, penicillin (100 μ/ml), streptomycin (100 μ/ml), and r-bFGF (10 ng/ml) in a 5% CO_2-humidified atmosphere. HUVECs were passed as a 1:5 split, and the medium was changed every 3 days. They were characterized as endothelial cells by their cobblestone appearance and positive staining for factor VIII antigen. HUVECs (3.0×10^4/well, fifth passage) were divided equally among four wells (Flexiperm®, Heraeus Instruments GmbH), in which glass cover slips were placed at the bottom. Cells were then cultured for 3 days in MCDB 131 containing 10% fetal bovine serum, penicillin (100 μ/ml), streptomycin (100 μ/ml), and r-bFGF(10 ng/ml) in a 5% CO_2-humidified atmosphere.

Incubation of Endothelial Cells With CLA and OA

Confluent culture cells grown on cover slips in Flexiperm® were rinsed once with HEPES-glucose buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 5 mM glucose, pH 7.4), then incubated with 20 nM CLA or 2 μM OA for 15 min at room temperature in the same buffer.

Morphological Study

The morphology was examined by means of phase contrast microscopy (OLYMPUS IMT-2) and scanning electron microscopy (SEM). For SEM, cells were fixed in phosphate buffer saline (PBS) containing 2.5% glutaraldehyde for 1 h on ice. The cells were post-fixed using 1% osmium tetraoxide in PBS for 30 min, then dehydrated through a graded ethanol series, and dried by the critical-point method using CO_2. The specimens were coated with platinum in a vacuum evaporator and examined under a HITACHI S-800 electron microscope at an accelerating voltage of 10 kV.

Immunocytochemistry

Cells were fixed in PBS containing 1% paraformaldehyde for 1 h on ice and permeabilized with 0.1% Triton X-100. Samples were rinsed with PBS and incubated for 30 min with mouse monoclonal antibodies against alpha-tubulin, phosphoserine, phosphothreonine, and phosphotyrosine. After rinsing with PBS, fixed cells were incubated for 30 min with fluorescein-conjugated goat anti-mouse IgG. Samples were then rinsed with PBS. To observe F-actin, cells were incubated for 30 min with rhodamine-phalloidin. Specimens were examined by confocal laser scanning microscopy (Zeiss LSM-410, Thornwood, NY).

Immunoblotting

After 15 min exposure to CLA at room temperature, 10 volumes of ice-cold HEPES-sucrose buffer (10 mM HEPES, 100 mM NaF, 10 mM EDTA, and 80 mM sucrose, pH 7.4) [Chartier et al., 1993] was added, then cells were scraped from the substratum with a rubber spatula and harvested by centrifugation at 100*g* for 5 min. Samples were washed twice with the HEPES-sucrose buffer, and dissolved in SDS sample buffer (2% SDS, 2% mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, 30 mM Tris, pH 6.8). After boiling for 5 min, the protein concentration was adjusted to 3 mg/ml with the SDS sample buffer. Samples (15 μl) were applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gels were electrophoretically transferred onto a PVDF protein sequencing membrane (0.2 μm pore size) [Towbin et al., 1979] for 60 min at 2 mA/cm^2 using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell apparatus (Bio Rad, Laboratories). The transferred membrane was incubated for 1 h at room temperature with blocking buffer (25 mM Tris-HCl, 154 mM NaCl, 2% BSA, pH 7.4), followed by an overnight incubation at 4°C with blocking

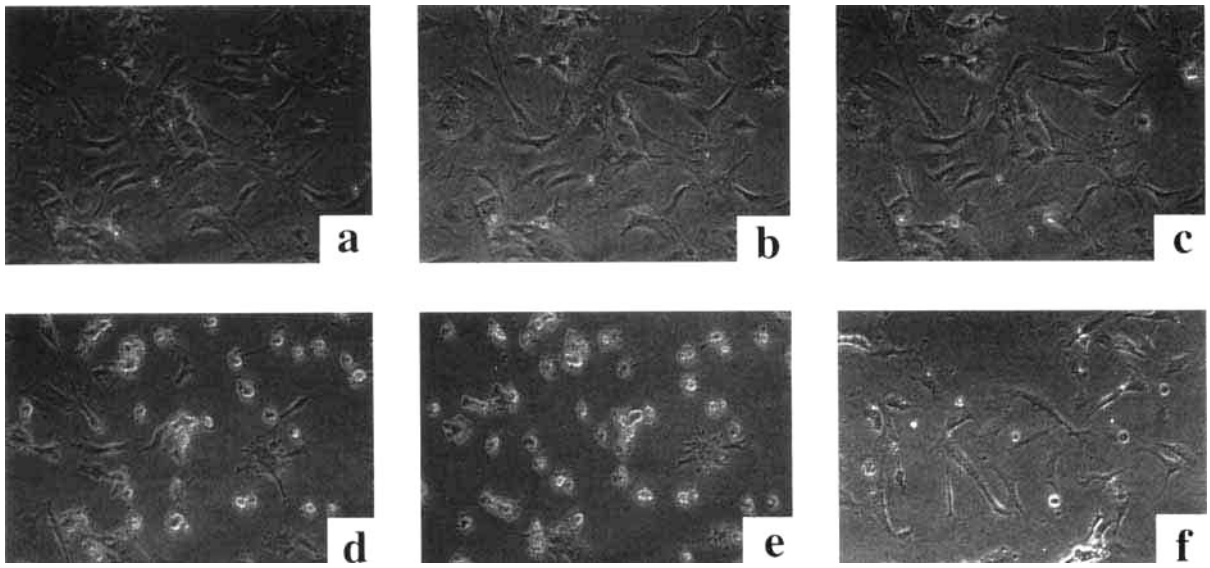

Fig. 1. *Phase contrast microscopy of control and CLA treated HUVECs. HUVECs cultured for 3 days were incubated* with 20 nM CLA. Cells were then examined by phase contrast microscopy. Control cells were incubated with glucose-HEPES buffer for 15 min (**a**). Cells were incubated with CLA for 1 (**b**), 5 (**c**), 10 (**d**), and 15 min (**e**). Incubation is with 1 μM K-252a for 1 min, followed by CLA for 15 min (**f**).

buffer containing a mouse monoclonal antibody. The membrane was washed three times with washing buffer (25 mM Tris-HCl, 0.25% gelatin, 0.5% Tween-80, pH 7.4), and developed with peroxidase-conjugated, goat anti-mouse IgG and 3,3′-diaminobenzidine tetrahydrochloride.

RESULTS

CLA (20 nM) induced significant morphological changes in cultured HUVECs when examined by phase contrast microscopy (Fig. 1). Cells became rounded within 5 min after the addition of CLA. This morphological change was mostly completed in 15 min. K252a (1 μM), a non-specific kinase inhibitor [Kase et al., 1989; Yasuzawa et al., 1986] abolished the effect of CLA. These findings were also confirmed by SEM (Fig. 2). There were numerous blebs on the cell surface of CLA treated cells (Fig. 2b). K252a completely inhibited not only the morphological change, but also bleb formation (Fig. 2c). Furthermore, the removal of CLA followed by an overnight incubation completely restored the normal shape of the HUVECs (data not shown). These results suggested that the morphological change induced by CLA is reversible and that it results from the inhibition of protein phosphatase activity.

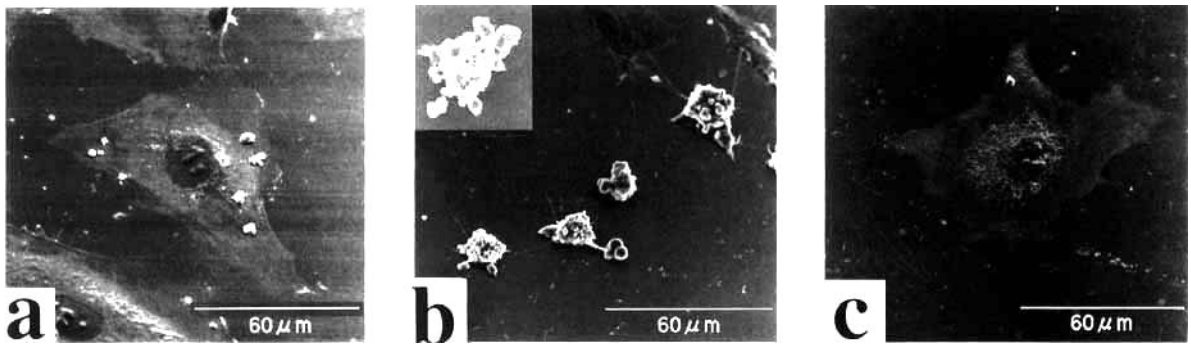

Fig. 2. Scanning electron microscopy (SEM) of control and CLA treated HUVECs. HUVECs were incubated with CLA (20 nM) as described in the legend to Figure 1. The cells were then fixed and dehydrated as described in the text. Control cells (**a**); CLA exposure for 15 min (**b**); incubation with K-252a (1 μM) for 1 min, followed by CLA for 15 min (**c**).

We investigated the intracellular mechanisms involved in these morphological changes. The structure of microtubules and F-actin, two major cytoskeletal components, was determined by confocal laser scanning microscopy, using rhodamine-phalloidin and a monoclonal antibody against alpha-tubulin (Fig. 3). F-actin and microtubules were distributed throughout the cytoplasm before CLA exposure (Fig. 3a and d). However, both of them became markedly condensed around the nucleus in the presence of CLA (Fig. 3b and e) and then were also seen in numerous surface blebs. Again, these effects of CLA were inhibited by K252a (Fig. 3c and f).

As these phenomena were considered to result from the phosphorylation of some specific proteins related to the cytoskeleton, protein phosphorylation was investigated by confocal laser scanning microscopy, using antibodies against phosphoserine, phosphothreonine, and phosphotyrosine (Fig. 4). In control cells, the cytoplasm and particularly the nucleolus, were markedly stained by anti-phosphoserine antibody (Fig. 4a). In contrast, the non-nucleolar area of the nucleus was significantly stained by antiphosphothreonine (Fig. 4b). The submembranous area was stained by anti-phosphotyrosine antibody (Fig. 4c). The staining profile of the nuclei was not altered by CLA with all antibodies, whereas that of the cytoplasm was markedly diminished in accordance with the morphological changes (Fig. 4d,e,f). The changes in protein phosphorylation by CLA were further investigated by Western blotting against these antibodies (Fig. 5). CLA significantly increased the phosphorylation levels of some specific proteins. Serine residues were phosphorylated in 135 and 158 kDa proteins. Threonine residues were phosphorylated in 140, 158, and 175 kDa proteins. In contrast, the tyrosine residue phosphorylation was not significantly different between cells with and without CLA.

CLA inhibits PP-1 almost 100 times more potently than OA, whereas both inhibitors are almost equally potent against PP-2A [Ishihara et al., 1989a]. Therefore, the morphological effects of these inhibitors were compared to elucidate which of PP-1 or PP-2A is related to the cytoskeletal reorganization. As shown in Figure 6, CLA was at least 100 times more potent in

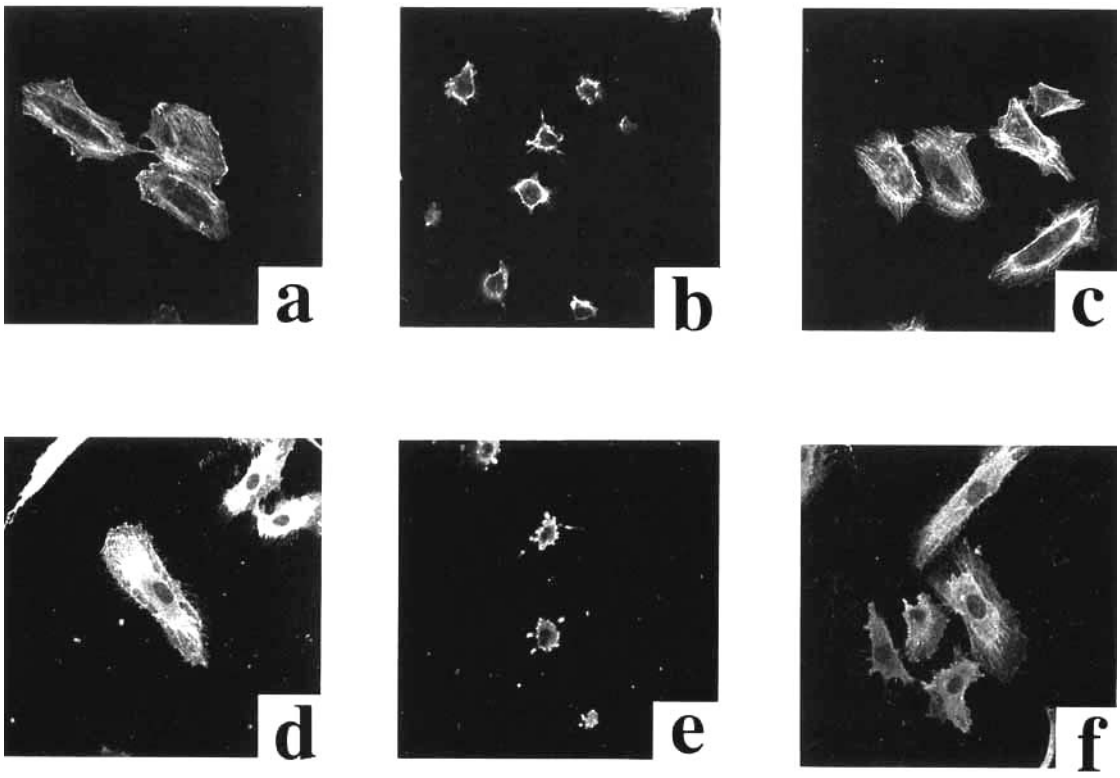

Fig. 3. Localization of F-actin and alpha-tubulin in control and CLA treated HUVECs. F-actin (**a–c**) and alpha-tubulin (**d–f**) were examined using rhodamine-phalloidin and a mouse anti-alpha tubulin monoclonal antibody as described in the text. Control (a, d). CLA (20 nM) (b, e). Incubation with K-252a (1 μM) followed by CLA (20 nM) for 15 min (c,f).

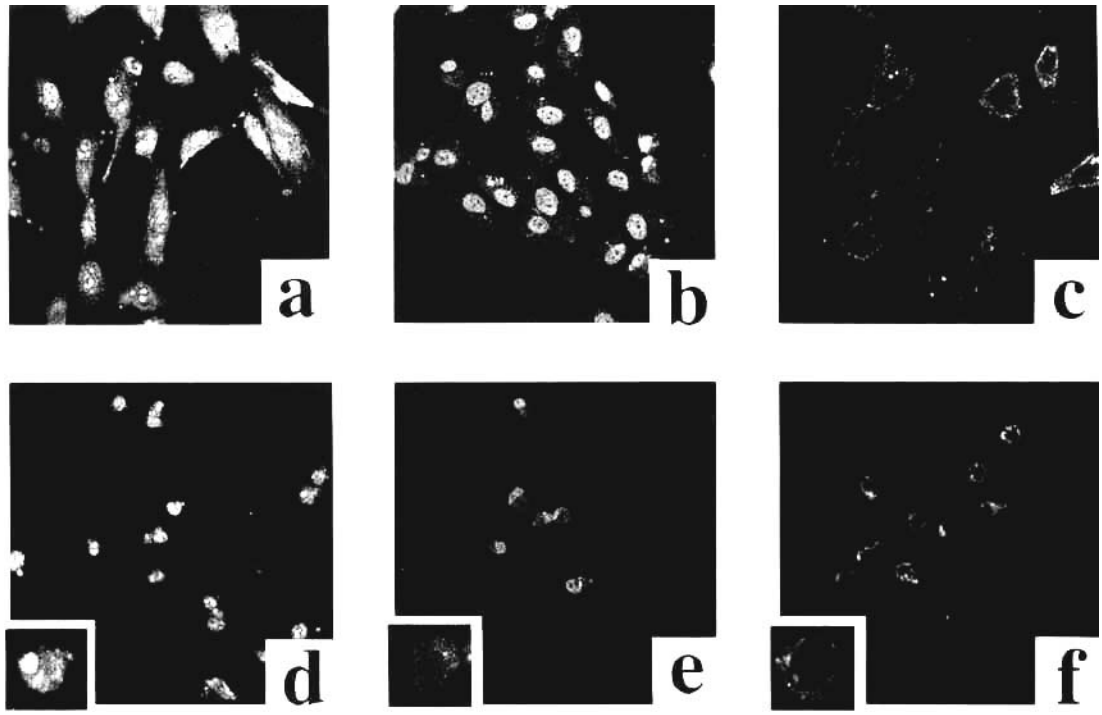

Fig. 4. Immunocytochemical study of CLA-induced protein phosphorylation in HUVECs. Intracellular protein phosphorylation was examined by immunocytochemistry with antibodies against phosphoamino acids. Control (**a,b,c**). CLA (20 nM) exposure for 15 min (**d,e,f**). Anti-phosphoserine antibody (a,d), anti-phosphothreonine antibody (b,e), anti-phosphotyrosine antibody (c,f).

inducing morphological changes than OA. These results suggest that PP-1, rather than PP-2A, is mainly involved in the restoration of cytoskeletal organization.

We confirmed the presence of PP-1 in HUVECs by Western blotting using an antibody against the catalytic subunit of PP-1 (Fig. 7). A single protein band (37 kDa) was detected, the molecular weight of which was identical with that reported for the catalytic subunit of PP-1 [Cohen, 1989].

DISCUSSION

CLA, a potent, cell-permeable inhibitor of serine/threonine protein phosphatase 1 and 2A (PP-1/PP-2A), induced significant morphological changes in cultured endothelial cells. These effects of CLA were not considered to be nonspecific because of the following reasons. Morphological changes induced by CLA were inhibited by the nonspecific protein kinase inhibitor, K252a (Figs. 1 and 2). Removal of CLA restored the cell shape (data not shown). The effect of CLA on plasma membrane fluidity or conformation is negligible when examined with ANS (1-anilino-8-naphthalene sulfate) and DPH (1.6-diphenyl-1,3,5-hexatriene) [Sakon et al., 1994]. Therefore, the CLA-induced morphological change in endothelial cells is likely to result from the inhibition of protein dephosphorylation. Consistent with findings in other cells (fibroblasts [Chartier et al., 1991; Hirano et al., 1992], neutrophils [Downey et al., 1993], smooth muscle cells [Hosoya et al., 1993; Ishihara et al., 1989a], F-actin and microtubules were redistributed around the nucleus or in membrane blebs following exposure to CLA (Fig. 3). These structural changes in the cytoskeleton were inhibited by K252a. Thus, it is conceivable that an unidentified protein kinase(s) persistently reorganizes F-actin and alpha-tubulin via the phosphorylation of some specific cytoskeletal proteins and that this effect of the protein kinase(s) is counteracted by a CLA-inhibitable protein phosphatase. Eriksson et al. have shown that CLA or OA induce the structural organization of intermediate filament and microtubules but not that of actin filament in BHK-21 fibroblasts, although an increasing number of cells showed a more rounded morphology when incubated with 20 μM CLA for more than 15 min [Eriksson et al., 1992]. The discrepancy in the morphological

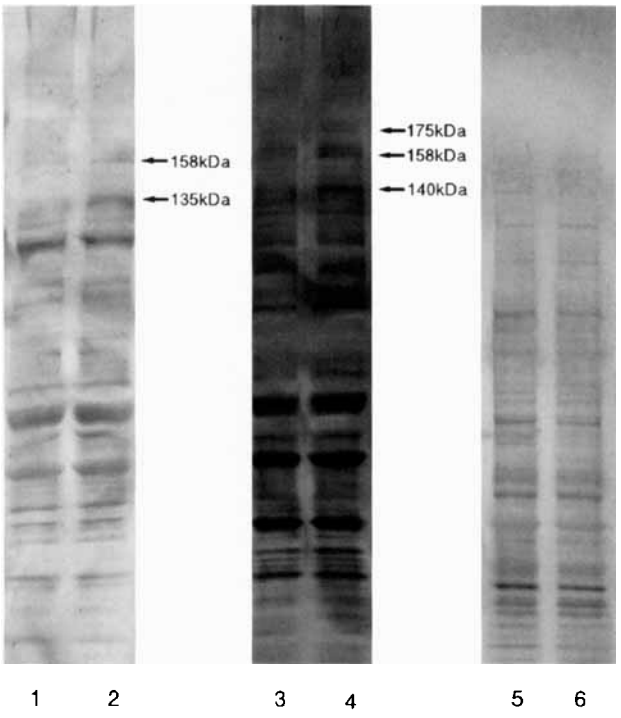

Fig. 5. Western blotting with antibodies against phosphoaminoacids. Cells incubated in the presence or absence of CLA were washed twice with HEPES-sucrose buffer, and dissolved in SDS-sample buffer as described in the text after electrophoresis (SDS-PAGE). Western blotting was performed using antibodies against phosphoserine (*lanes 1* and *2*), phosphothreonine (*lanes 3* and *4*), and phosphotyrosine (*lanes 5* and *6*). Control (lanes 1, 3, and 5); HUVECs incubated with CLA (lanes 2, 4, and 6).

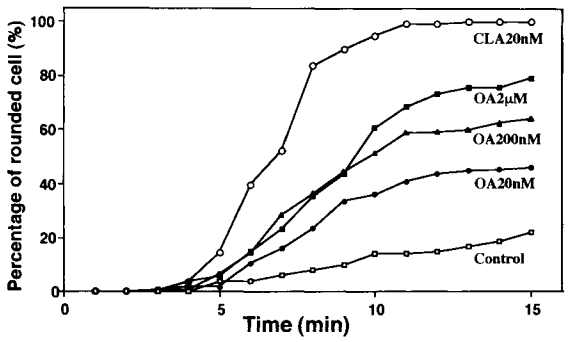

Fig. 6. The differential effects of CLA and OA on cell morphology of HUVECs. After the addition of phosphatase inhibitors, the cells were counted under phase contrast microscopy and the percentage of rounded cells was calculated.

changes may be due to the different permeability of CLA. Gurland and Gundersen also demonstrated that these phosphatase inhibitors caused the breakdown of the stable microtubules (MTs) enriched in posttranslationally detyrosinated tubulins (Glu-MTs) in 3T3 fibroblasts, but did not observe morphological changes even after a 60 min incubation with 1 μM OA [Gurland and Gundersen 1993]. As the cell shape is generally considered to be regulated by the both microtubule and actomyosin systems, the contradictory results between Gurland and Gundersen's work and ours may be explained by the different effect on the actomyosin system in endothelial cells and fibroblasts.

CLA specifically inhibits only PP-1 and PP-2A [Ishihara et al., 1989b]. Consistent with these findings, CLA did not affect tyrosine phosphorylation in HUVECs when whole cells were examined by Western blotting with anti-phosphotyrosine antibody, while serine or threonine phosphorylation was significantly increased in some specific proteins (Fig. 5). These results suggested that serine/threonine, but not tyrosine phosphorylation, is responsible for CLA induced cytoskeleton reorganization. As shown in Figure 6, CLA induced morphological changes more than 100 times potently than OA. The IC_{50} of CLA for PP-1 (2 nM order) is about 100 times less than that of OA (224 nM order), whereas that for PP-2A is almost identical between these inhibitions [Ishihara et al., 1989b]. Thus, these dose-dependent effects on morphological changes were comparable to those on PP-1, but not on PP-2A. Therefore, PP-1, rather than PP-2A,

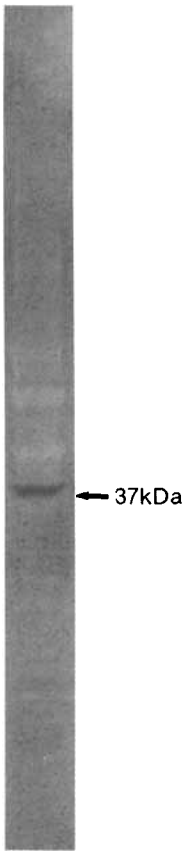

Fig. 7. Cultured HUVECs were washed twice with HEPES-sucrose buffer, dissolved in SDS sample buffer, then Western blotted using an antibody against the catalytic subunit of PP-1.

plays a predominant role in sustaining the normal cytoskeletal structure. Although PP-1 has been identified in various cells and tissues [Hubbard and Cohen, 1993], its presence in endothelial cells has not been studied so far. We confirmed for the first time, the presence of PP-1 in endothelial cells, by means of Western blotting using an antibody against its catalytic subunit (Fig. 7).

Western blotting with antibodies against these phosphoamino acids demonstrated that polypeptides of 135, 140, 158, and 175 kDa were significantly phosphorylated on serine and/or threonine residues, although such effect on tyrosine phosphorylation was not observed. These phosphoproteins, including their amino acid sequence are now being characterized to clarify their possible roles in cytoskeletal reorganization. The serine/threonine protein kinase(s) involved in the phosphorylation of these polypeptides also remains unknown. However, as these polypeptides were phosphorylated in the presence of phosphatase inhibitor alone, they are considered to be persistently active. The molecular mechanisms involved in the reorganization of actin filament by protein phosphorylation and dephosphorylation remain unknown. As CLA does not activate platelets [Murata et al., 1992], the constriction of the cell body following exposure to CLA might be explained by the ATP or Ca^{2+} independent constriction of actomyosin, namely the "latch phenomenon" [Aksoy et al., 1983; Chatterjee and Murphy, 1983], although its molecular mechanisms remain unknown. Some regulatory proteins in the actomyosin system, such as MARCKS (myristoylated alanine-rich C kinase substrate), calponin, and caldesmon are phosphorylated and thereby may be implicated in this state. MARCKS, an actin binding protein, is phosphorylated by serine/threonine kinase(s) like C-kinase [Aderem, 1992]. Phosphorylated MARCKS is released from the membrane, but it binds to the membrane again after dephosphorylation [Wang et al., 1989; Thelen et al., 1991; Hartwig et al., 1992]. Calponin is phosphorylated by protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II. The phosphorylation of calponin by either protein kinase C or Ca^{2+}/calmodulin-dependent protein kinase II abolishes the inhibitory effects of calponin against actomyosin ATPase activity [Winder and Walsh, 1990; Nakamura et al., 1993]. Since PP-1 dephosphorylates calponin [Okubo et al., 1994], the constriction of F-actin by CLA may result from the inhibition of calponin dephosphorylation. Talin, one of the anchoring proteins between actin filaments and integrins, is phosphorylated on threonine residues by CLA [Murata, 1995]. Its affinity for actin filament or integrins drastically decreased, which may contribute to the redistribution of actin filaments. Myosin light chain (MLC) phosphatase is a PP-1 [Ishihara, 1989b]. However, in this study, MLC was not significantly phosphorylated by CLA (Fig. 5). Similar results were also obtained in platelets [Yano et al., unpublished data]. These findings differ from those reported by Chartier et al. [1991]. They showed that a 15 min incubation with CLA increased the phosphorylation of MLC in 3T3-fibroblasts, although they used 100 nM CLA, a concentration five times higher than that used in this study. Thus, various mechanisms via protein dephosphorylation may be involved in the cytoskeletal stabilization of endothelial cells. This may be better elucidated in the near future by characterizing

the phosphoproteins (135, 140, 158, and 175 kDa) demonstrated in this study. In conclusion, serine/threonine protein PP-1 may be involved in the regulation of cytoskeletal structure by dephosphorylating some specific phosphoprotein.

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