Journal of Cellular Biochemistry 63:311–319 (1996)

Protein Phosphatase 2A in Stretch-Induced Endothelial Cell Proliferation

Kohei Murata, Ira Mills, and Bauer E. Sumpio

Department of Surgery, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract We previously proposed that activation of protein kinase C is a key mechanism for control of cell growth enhanced by cyclic strain [Rosales and Sumpio (1992): Surgery 112:459–466]. Here we examined protein phosphatase 1 and 2A activity in bovine aortic endothelial cells exposed to cyclic strain. Protein phosphatase 2A activity in the cytosol was decreased by 36.1% in response to cyclic strain for 60 min, whereas the activity in the membrane did not change. Treatment with low concentration (0.1 nM) of okadaic acid enhanced proliferation of both static and stretched endothelial cells in 10% fetal bovine serum. These data suggest that protein phosphatase 2A acts as a growth suppressor and cyclic strain may enhance cellular proliferation by inhibiting protein phosphatase 2A as well as stimulating protein kinase C. © 1996 Wiley-Liss, Inc.

Key words: protein phosphatase 2A, endothelial cells, cyclic strain, proliferation, okadaic acid

Endothelial cells (EC) play a central role in atherogenesis and thrombosis. EC response to neurohumoral factors and hemodynamic forces includes alterations in vascular permeability, vasomotor tone, and proliferation. A number of mitogenic factors, including epidermal growth factor, basic fibroblast growth factor, thrombin, and insulin, and mechanical forces, such as cyclic strain and pressure, are known to induce proliferation of EC [Sumpio et al., 1987; Oliveila and Banerjee, 1990; Hawker and Granger, 1994]. However, very little is known about the intracellular mechanisms that regulate proliferation of EC in response to those mechanical forces.

Protein phosphorylation is an important regulatory mechanism for a variety of cells. It is the resultant balance between kinase and phosphatase activity. Several studies underscore this importance. For example, EC protein phosphorylation can be stimulated by the protein kinase C (PKC) and calcium/calmodulin kinase pathways [Mackie et al., 1986; Demolle et al., 1988]. PKC can also mediate bidirectional DNA synthesis in EC through phosphorylating retinoblastoma protein [Zhou et al., 1993]. Although they investigated kinases in detail, these investigators did not mention phosphatases.

Protein phosphatase (PP) 1 and 2A dephosphorylate phosphorylated serine and threonine residues and have been implicated in the regulation of cellular proliferation and phenotype [Shenolikar and Nairn, 1991; Mumby and Walter, 1993]. Although a report suggests the importance of protein phosphatases that dephosphorylate the P29 protein, which is phosphorylated by IL-1 treatment of EC [Levin and Santell, 1991], there is little information about protein phosphatase levels in EC and minimal information about their importance in EC function [Verin et al., 1995].

Our laboratory has reported previously that PKC in the membrane fraction of EC lysate is activated in response to cyclic strain [Rosales and Sumpio, 1992]. To understand cellular responses that are dependent on the phosphorylated state of key proteins that are unknown, it is also important to examine the role of protein phosphatase in EC exposed to cyclic strain. The aim of this study is to measure protein phosphatase 1 and 2A activity in EC exposed to cyclic strain and to examine the involvement of PP1 and/or 2A in the stretch-induced proliferation of EC using a specific protein phosphatase inhibitor, okadaic acid.

Received January 9, 1996; accepted May 16, 1996.

Address reprint requests to Dr. Bauer E. Sumpio, Department of Surgery, Yale University School of Medicine, 333 Cedar Street, FMB 137, New Haven, CT 06510.

Kohei Murata's present address is Center for Cell Signaling, University of Virginia, Box 577, Health Sciences Center, Charlottesville, VA 22908.

MATERIALS AND METHODS

Materials

Phosphorylase b and phosphorylase kinase were purchased from GIBCO BRL (Gaithersburg, MD). Okadaic acid (OA) was obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from HyClone laboratories (Logan, UT). [γ-32P]ATP and [methyl-^{3}H]thymidine were purchased from New England Nuclear (NEN) (Boston, MA).

Cell Culture

Bovine aortic endothelial cells (BAEC) were harvested and cultured as described previously [Rosales and Sumpio, 1992]. The cells were seeded on type 1 collagen-coated Silastic membranes (Flexcell, McKeesport, PA) and maintained in a humidified atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (GIBCO BRL) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 5 μg/ml deoxycytidine, 5 μg/ml thymidine, and 10% (v/v) FBS. Cells were grown in 10% FBS because cultured BAEC could be maintained only in this condition when they were stretched in our system.

Application of Cyclic Strain

Cells were exposed to cyclic strain as described and characterized in detail before [Sumpio et al., 1987; Rosales and Sumpio, 1992]. The apparatus consists of computer-controlled pressure transducers and solenoid valves that precisely control the duration, frequency, and magnitude of vacuum applied to the bottoms of six-well plates with flexible silicone membranes coated with type I collagen (Flex I, Flexcell). BAEC were seeded onto Flex I plates and placed in a stress unit in a CO_2 incubator (5% CO_2, 37°C). EC attached to the membrane were exposed to 150 mm Hg vacuum at 60 cpm (0.5-sec strain, 0.5-sec relaxation; 10% average strain).

Cellular Fractionation

BAEC were incubated in medium containing 10% FBS. After 30-min cyclic strain, medium was removed and cells washed twice with ice-cold phosphate-buffered saline (PBS). BAEC were lysed by two different methods. In the first method, we examined the activity of PP1 and PP2A in detergent-soluble and -insoluble fractions. EC were lysed in wells using ice-cold buffer A (20 mM Tris–HCl pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 250 mM sucrose) containing 0.1% Triton X-100. The supernatants were collected as Triton-soluble fractions. Triton-permeabilized cells were washed and scraped, centrifuged at 10,000*g* for 5 min at 4°C, and washed with buffer A. The pellets were resuspended by vortex in buffer A containing 0.5 M KCl and kept on ice for 1 h to extract cytoskeletal proteins. The suspensions were centrifuged at 10,000*g* for 5 min at 4°C. The supernatants contained the Triton-insoluble (cytoskeletal protein) fractions.

In the second method, we separated cytosol and membrane fractions. EC were scraped, sonicated briefly in buffer A on ice, and centrifuged at 100,000*g* for 1 h at 4°C. The supernatants were collected as cytosol fractions, and pellets were washed and solubilized with buffer A containing 0.1% Triton-X 100. The solubilized pellets were centrifuged at 100,000*g* for 1 h at 4°C, and the supernatants were collected as membrane fractions.

Protein Phosphatase 1 and 2A Activities in BAEC

Protein phosphatase 1 and 2A activities were measured as described by Brautigan and Shriner [1988] using ^{32}P-labeled-phosphorylase a as a substrate. Phosphorylase b was converted to ^{32}P-labeled-phosphorylase a with phosphorylase kinase and 0.5 mCi of [γ-^{32}P]ATP. Each sample (20 μl) from different cell fractions was mixed with 20 μl of the reaction buffer (20 mM imidazole–HCl, pH 7.63, 0.2 mM EDTA, 15 mM β-mercaptoethanol, 1 mg/ml BSA) containing okadaic acid or vehicle (0.1% ethanol). The reaction was initiated by adding 20 μl of 3 mg/ml ^{32}P-labeled-phosphorylase a. After incubation at 30°C for 10 min, 540 μl of ice-cold 20% trichloroacetic acid (TCA) was added and samples were centrifuged at 10,000*g* for 10 min at 4°C. Supernatant (500 μl) was transferred into 3 ml of deionized water, and Chelenkov counting was done by scintillation counter. Each count was less than 30% of total cpm to achieve linear correlation between cpm and activity. Specific activity was defined as nmoles of Pi release/min/mg protein. PP1 activity was determined by adding 1 nM OA to inhibit PP2A activity. PP2A activity was obtained by subtracting the amount of activity remaining in the presence of 1 nM OA from the activity without OA [Huang et al., 1995]. Protein concentration was measured according to Bradford's

method using BSA as the standard [Bradford, 1976].

Assay for Cell Proliferation

BAEC (50,000/well) were seeded and cultured in 10% FBS medium; 12 h after seeding (day 0), medium was replaced with medium containing OA. Cells were exposed to cyclic strain as described above or put in the same incubator as control. The medium was changed every 48 h with or without OA. Cell number was measured by Coulter counter analysis at day 1, 3, 5, 7 (24 h after medium change) as described previously [Rosales and Sumpio, 1992].

DNA synthesis BAEC was determined by measuring [^{3}H]thymidine incorporation by a slight modification of the method described by Wilson et al. [1993]. BAEC with or without OA (0.1 nM) were seeded (100,000/well) and incubated in 10% FBS medium. After 18 h, 2 mCi [^{3}H]thymidine was added to each well, and cells were incubated for an additional 6 h. Medium was aspirated and wells were washed twice with PBS containing 2 mM $CaCl_2$. Then, cells were extracted with ice-cold 15% TCA, kept at 4°C for 60 min, and washed with ice-cold 15% TCA. The well bottoms were removed and left in water-soluble scintillation liquid at room temperature overnight. Radioactivity was measured with a scintillation counter. For morphological analysis, BAEC were viewed by phase-contrast microscopy (Olympus IMT-2) and photographed using an automatic exposure system.

Statistics

All the data are shown as mean ±SE. The data were subjected to analysis of variance (ANOVA) with post hoc testing. Paired t-test was used for the phosphatase activity in stretched cells, with $P < 0.05$ considered significant.

RESULTS

Protein Phosphatase 1 and 2A Activity in Stretched-BAEC

In detergent (Triton X-100) fractionation of BAEC, basal phosphorylase a phosphatase activity (protein phosphatase 1 and 2A activity) was 60.9 ± 2.9% of total activity in the soluble fraction and 39.1 ± 2.9% in the insoluble fraction. However, specific activity was three times higher in the insoluble fraction than in the soluble fraction (Table I). In sonicated cell extracts, activity was located predominantly in the cytosol fraction (98.9 ± 2.3% of total activity) compared to the membrane fraction (1.1 ± 0.23%). Specific activity was also more than 10 times higher in the cytosol fraction than in the membrane fraction (Table I). The IC_{50} of OA for purified PP1 is known to be 10–20 nM and for purified PP2A 0.05–0.10 nM in vitro [Bialojan and Takai, 1988]. Therefore, it can be determined which phosphatase, 1 or 2A, is predominant in each fraction of BAEC by adding OA in vitro. As shown in Figure 1, the IC_{50} of OA for the Triton-insoluble fraction was less than 0.1 nM. On the other hand, the IC_{50} of OA for the soluble fraction was about 10 nM. These data indicate that in the Triton-insoluble fraction of BAEC, PP2A is predominant but in the soluble fraction, PP1 is predominant. IC_{50} values of both cytosolic BAEC phosphatase and membrane activity were around 1 nM, indicating that cytosol and membrane exhibit both PP1 and PP2A activity to a considerable amount.

When cyclic strain was applied to BAEC for 30 min, no significant change in phosphatase activity was seen in Triton fractionation (Fig. 2). However, in the cytosol fraction of sonicated extract, PP2A activity was significantly decreased by 36.1%, while in the membrane fraction no significant increase was observed (Fig. 3).

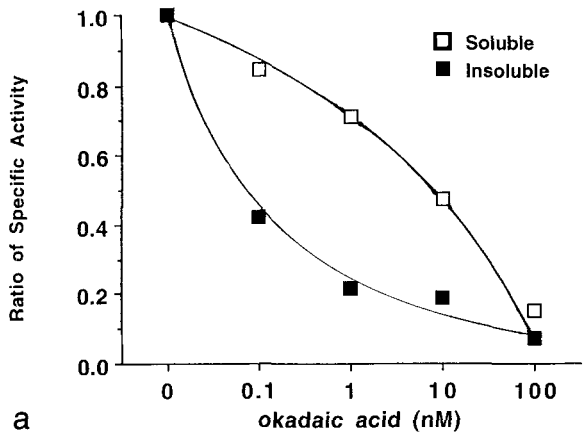
TABLE I. Phosphorylase a Phosphatase Activity of Bovine Aortic Endothelial Cells*

	Triton-X 100 fractionation		Sonication fractionation	
	Soluble	Insoluble	Cytosol	Membrane
Specific activity[a] (nmol/min/mg)	6.05 ± 0.82	18.64 ± 1.21	27.94 ± 5.58	2.20 ± 0.33
Total activity[b] (%)	60.9 ± 2.9	39.1 ± 2.9	98.9 ± 2.3	1.1 ± 0.2

*BAEC were lysed with 0.1% Triton-X 100 and fractionated into soluble and insoluble fractions or sonicated into cytosol and membrane fractions, as described under Materials and Methods. Phosphorylase a phosphatase activity was determined using ^{32}P-labeled phosphorylase a as a substrate for both PP1 and PP2A.

[a]Specific activity was defined as released Pi nmoles per minute per mg protein.

[b]Total activity (%) was the ratio of the amount of activity of either soluble versus insoluble, or cytosol versus membrane. Values represent the mean ±SE of four experiments.



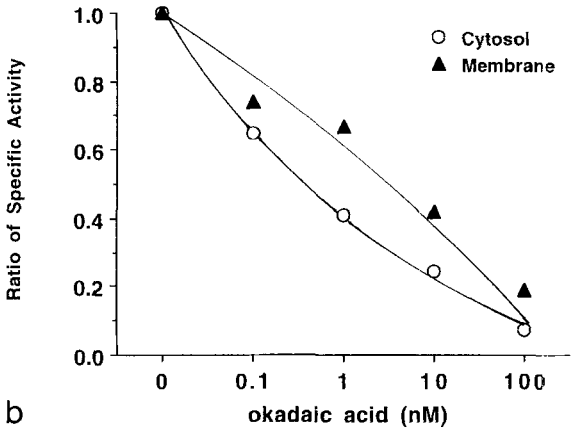
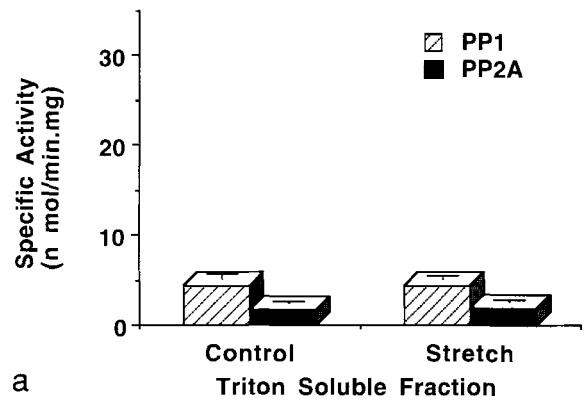


Fig. 1. Inhibition of phosphorylase a phosphatase activity in bovine aortic endothelial cells (BAEC) by okadaic acid (OA). BAEC were lysed with 0.1% Triton-X 100 or sonicated. Lysates were then fractionated into Triton-soluble and -insoluble, or cytosol and membrane fraction as described in Materials and Methods. Samples were incubated with different concentrations of OA. **a:** *Triton-soluble and -insoluble fraction.* **b:** *Cytosol* and membrane fraction. OA was added in vitro before reactions. Inhibition of specific activity of phosphorylase a phosphatase is shown as ratio of the control values observed in the absence of OA. Data shown are from one representative set of samples. Similar inhibition curves were obtained from three other experiments.



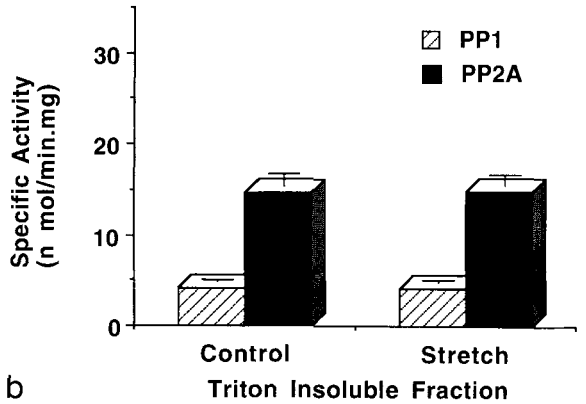


Fig. 2. Protein phosphatase (PP) 1 and 2A activity in Triton-soluble and -insoluble fractions of bovine aortic endothelial cells (BAEC) under static (control) or stretched (stretch) condition. BAEC were cultured in 10% fetal bovine serum medium and stretched for 30 min, as described under Materials and Methods. Activities of PP1 and 2A of (a) Triton-soluble fraction and (b) Triton-insoluble fraction were measured using ^{32}P-labeled phosphorylase a as a substrate for both PP1 and PP2A. PP1 activity was determined at 1 nM okadaic acid (OA) to inhibit PP2A activity and PP2A activity was determined as a subtraction of PP1 activity from total phosphorylase a activity. Specific activity was defined as released Pi nmoles per minute per mg protein. Data are mean ±SE from four individual experiments.

Effects of Low Concentration of Okadaic Acid on Proliferation and [^{3}H]Thymidine Incorporation of Stretched-BAEC

Since OA is known to be cell permeable, we applied OA to intact BAEC to determine the involvement of PP1 or 2A in stretch-enhanced cellular proliferation. First, we tested the effect of OA on cell toxicity. Treatment with low doses (0.1–10 nM) of OA for 24 h did not affect cell shape. However, exposure to 100 nM OA for 24 h caused BAEC detachment (Fig. 4). This effect was reversible, since BAEC reattached after washing out the OA (data not shown). Three-day treatment with 10 nM OA also led to reversible cell detachment. OA (0.1 and 1 nM) did not affect cell shape or attachment until 7 days of treatment in static conditions, but some cells were detached at day 5 of 1 nM OA in the stretched condition. These results indicate that only 0.1 nM of OA should be used for the 7-day cell proliferation studies in stretched condition. No significant change in cell number was observed between vehicle and OA treatment during the first 3 days, whereas cyclic strain en-

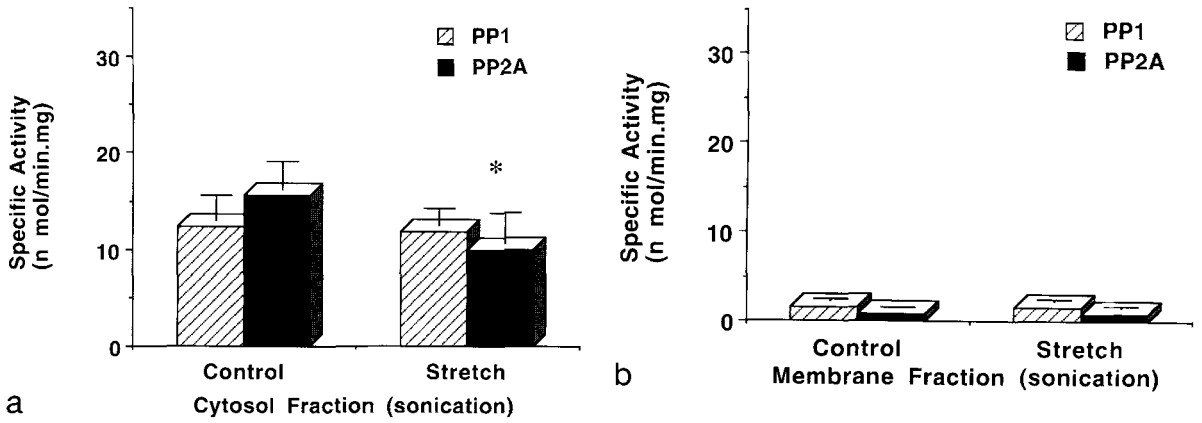


Fig. 3. Protein phosphatase (PP) 1 and 2A activity in cytosol and membrane fractions of bovine aortic endothelial cells (BAEC) under static (control) or stretched (stretch) condition. BAEC were cultured in 10% fetal bovine serum medium and stretched for 30 min as described under Materials and Methods. Activities of PP1 and 2A of (**a**) cytosol fraction and (**b**) membrane fraction were measured using ^{32}P-labeled phosphorylase a as a substrate for both PP1 and PP2A. PP1 activity was determined at 1 nM okadaic acid (OA) to inhibit PP2A activity and PP2A activity was determined as a subtraction of PP1 activity from total phosphorylase a activity. Specific activity was defined as released Pi nmoles per minute per mg protein. Data are mean ±SE from four individual experiments. *$P < 0.05$ (vs control), paired t-test.

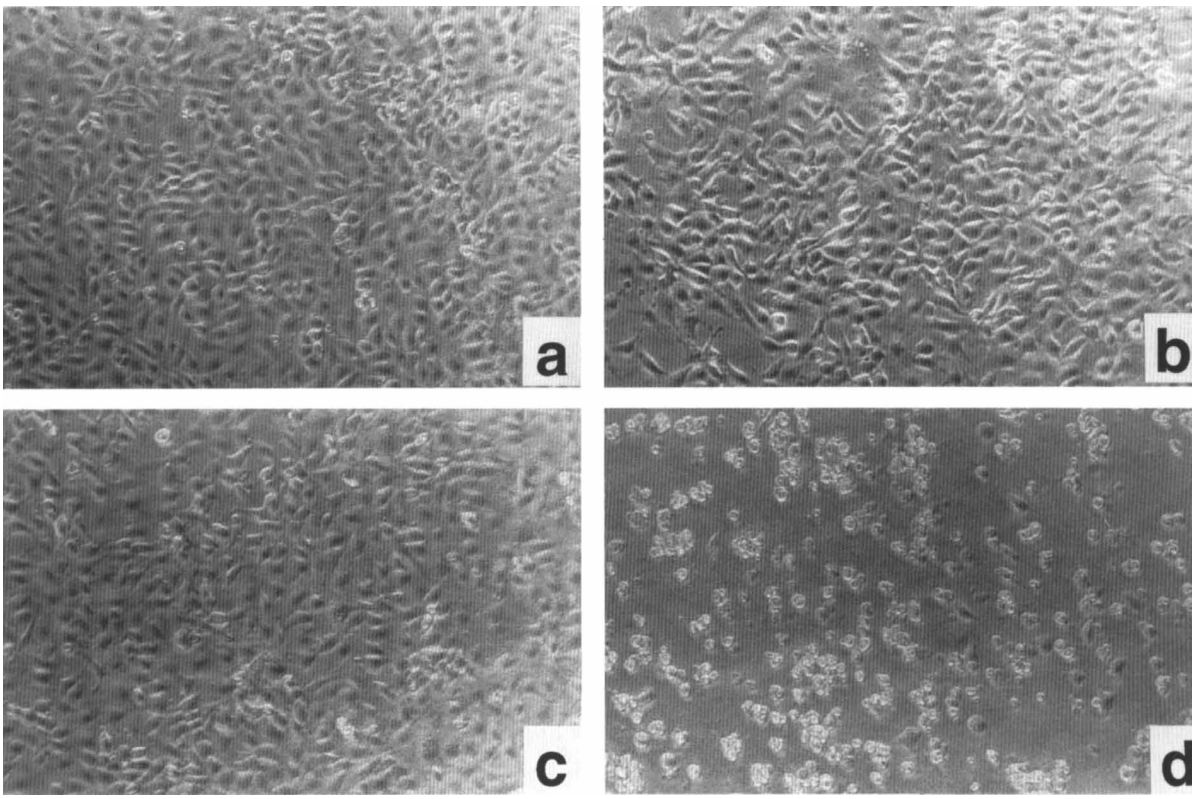


Fig. 4. The effect of okadaic acid (OA) on morphology of bovine aortic endothelial cells (BAEC). BAEC were seeded onto Flexcell membranes and cultured in the medium containing 10% fetal bovine serum with various concentrations of okadaic acid (OA). **a:** Vehicle. **b:** OA 1 nM. **c:** OA 10 nM. **d:** OA 100 nM. After 24-h incubation at 37°C in CO_2 incubator, photographs were obtained by phase-contrast microscopy (×150). Some cells were out of focus because Flexcell was concave.

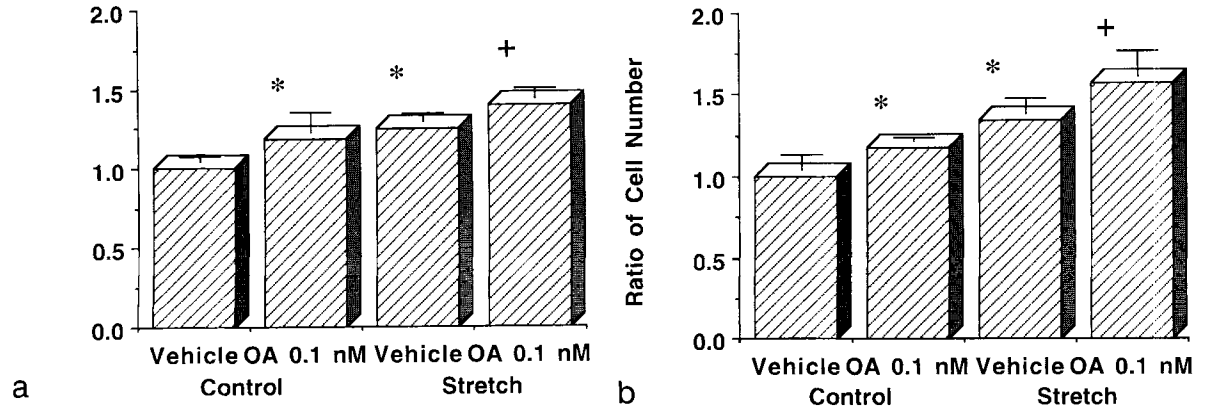


Fig. 5. Effect of okadaic acid (OA) on stretch-induced proliferation of bovine aortic endothelial cells (BAEC). BAEC were cultured for up to 7 days in 10% fetal bovine serum medium with okadaic acid (OA) (0.1 nM) or 0.1% ethanol as vehicle under static (control) or stretched (stretch) condition. Culture medium was changed every 48 h with fresh OA. Cell number was determined as described under Materials and Methods at day 5 (**a**) and day 7 (**b**). Data are mean ±SE from three determinations. $*P < 0.05$ (vs vehicle in control), $+P < 0.05$ (vs vehicle in stretch), Student's *t*-test. Similar results were obtained from three different batches of cells.

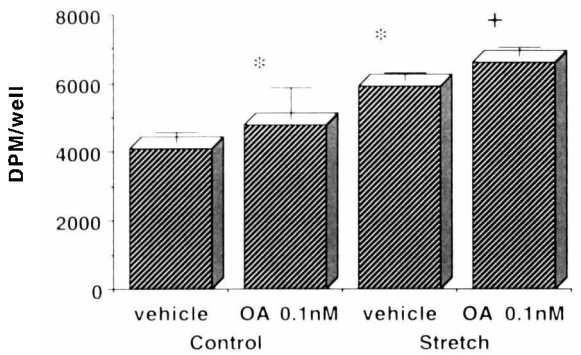


Fig. 6. [^{3}H]Thymidine uptake in bovine aortic endothelial cells (BAEC). BAEC were incubated in 10% fetal bovine serum medium with okadaic acid (OA) (0.1 nM) or 0.1% ethanol as vehicle. After 18-h incubation in CO_2 incubator at 37°C, 2 mCi [^{3}H]thymidine was added to each well, and cells were incubated for additional 6 h under static (control) or stretched (stretch) condition. Thymidine incorporation into DNA was determined as described under Methods and Materials. Data are mean ±SE from five wells. $*P < 0.05$, vs vehicle in control, $+P < 0.05$, vs. vehicle in stretch, Student's *t*-test. Similar results were obtained from two different batches of cells.

hanced cell proliferation [Rosales and Sumpio, 1992]. However, 0.1 nM OA significantly enhanced cell number of both static and stretched BAEC at day 5 (Fig. 5a) and day 7 (Fig. 5b).

As shown in Figure 6, FBS-induced DNA synthesis in both static and stretched BAEC measured with [^{3}H]thymidine incorporation was enhanced after 24-h incubation with 0.1 nM OA.

DISCUSSION

Cyclic strain has been demonstrated to enhance serum-induced EC proliferation [Sumpio et al., 1987]. Although the mechanisms responsible for this enhancement are not fully clarified, PKC activity has been shown to translocate from cytosol to membrane after a 10% average stretch at 60 cpm for 500 sec [Rosales and Sumpio, 1992]. We have also shown that treatment with calphostin C, a specific PKC inhibitor, abrogated stretch-induced increase in [^{3}H]thymidine incorporation. Since phosphorylation is a reversible reaction and both kinase and phosphatase activities are known to be modified in response to various stimuli, phosphatase activity in stretched cells might be altered.

Only a few papers have appeared concerning protein phosphatase in EC [Demolle et al., 1988; Levin and Santell, 1991]. Recently, Verin et al. [1995] reported that both PP1 and PP2A are constitutively active toward two distinct substrates, myosin light chain and phosphorylase a, although specific activity of PP1 or PP2A was not shown. We used [^{32}P]phosphorylase a as a substrate for both PP1 and PP2A. If 1 nM OA is used in the assay, only PP2A is inhibited. Thus, 1 nM OA resistant activity can be interpreted to PP1 activity. PP2A activity is obtained indirectly by subtracting PP1 activity from full activity. This differential measurement of PP1 and PP2A is applied for other cells including neuronal cells [Cohen et al., 1989; Huang et al., 1995].

When Triton X-100 was used for cell lysis, the Triton-insoluble fraction was more sensitive to 1 nM OA. These data implicated that in the detergent-insoluble fraction, PP2A activity is greater than PP1 compared to the detergent-

soluble fraction of BAEC. PP2A is shown to be associated with microtubles and to play a critical role during cell cycle [Sontag et al., 1995]. PP2A in actin filament is also known to be involved in bud growth [Lin and Arndt, 1995]. These findings suggest a high basal activity of PP2A in cytoskeletal proteins. High level of PP2A specific activity in BAEC cytoskeleton implies the significance of this enzyme in cytoskeletal protein, although we did not see a significant change by stretch.

In the sonicated cells, BAEC displayed greater phosphorylase a phosphatase activity in the cytosol fraction, as compared with other types of normal cultured cells, including fetal chick neurons [Begum et al., 1993], or murine T cells [Matsuzawa et al., 1993]. At the sonication step in our method, some of cytoskeletal proteins may be also extracted in this cytosol fraction. Specific activity of PP1 or PP2A was not higher in the membrane fraction than the cytosol of BAEC, as reported previously for PKC [Rosales and Sumpio, 1992], suggesting that PP1 and PP2A act mainly in cytosol. PP2A activity in the cytosol fraction decreased significantly by stretch. The change was as small as 36.1%, compared with PKC activation (77.8%), reported previously. The reason why we could not see a large difference of PP2A activity in response to stretch may be because phosphorylase a is not the best substrate with which to detect phosphatase activity responsible to stretch. Although this change did not appear to be caused by translocation of PP2A from membrane to cytosol-like PKC activity, as shown by Rosales and Sumpio [1992], it suggests that PP2A is also modified by mechanical stretch as well as by PKC.

PP2A is thought to play an important role in regulating cell growth control. In amphibian oocytes, PP2A dephosphorylates maturation promoting factor; this event correlates with a functional loss of maturation [Ruediger et al., 1991]. In addition, several physiological substrates for cyclin-dependent kinases have been demonstrated to be dephosphorylated by PP2A [Ferrigno et al., 1993]. PP2A can be inhibited by SV40 small T antigen; this inhibition is thought to explain the growth-promoting effect of small T antigen, which occurs through the MAP kinase pathway [Sontag et al., 1993]. In BAEC, cyclic strain may inhibit PP2A activity to enhance serum-induced proliferation; this inhibition may induce hyperphosphorylation of unknown proteins, probably not substrates for PKC. Recent reports showed that PP2A is inhibited through tyrosine phosphorylation in serum-induced proliferation of fibroblasts [Chen et al., 1992, 1994]. These reports suggest that PP2A plays a role as a growth suppressor, and inhibition of PP2A is required for cellular proliferation. Our results are consistent with this idea.

In the present study, serum-induced cell proliferation of both static and stretched BAEC was enhanced in the presence of 0.1 nM OA, which has an inhibitory effect on phosphatase activity of BAEC in vitro, as shown in Figure 1. This concentration is known to inhibit only PP2A [Bialojan and Takai, 1988; Cohen et al., 1992]. OA is a potent tumor promoter, and it has been suggested that this phosphatase inhibitor can stimulate cellular proliferation [Walter and Mumby, 1993]. By contrast, a dose-dependent growth inhibitory effect of OA has been reported in various cell types under a variety of conditions [Ishida et al., 1992; van Dolah and Ramsdell, 1992; Sakurada et al., 1992; Rieber and Rieber, 1993]. OA has been reported to inhibit cellular transformation and transforming growth factor-b (TGF-b)- or 10% serum-induced proliferation in quiescent postconfluent C3H/10T1/2 cells [Kim et al., 1993]. The inhibition or stimulation of cell proliferation by OA might be cell specific. However, experimental conditions, including drug concentration and incubation time, are also important. High concentrations of OA induce strong phenotypic and molecular changes in cells and may obscure the growth stimulatory effects observed at low concentrations because higher dose inhibits both PP1 and PP2A, whereas lower dose inhibits only PP2A [Walter and Mumby, 1993]. For example, treatment of BHK-21 fibroblasts with 500–1,000 nM OA for 60 min causes cells to round up and induces cytoskeletal alteration [Eriksson and Golman, 1993]. Although this phenomenon is reversible and does not necessarily result in cell death, cell detachment from substrate will affect proliferation. Furthermore, OA may not be evenly distributed in the whole cell within a short incubation time, although it is shown to be cell permeable. The effects of prolonged with OA have not yet been fully examined. Thus, it is important to define an optimal dose and incubation time of OA that do not affect cell morphology or attachment, when determining their effects on proliferation. We examined the effects of OA at different doses on BAEC morphology and

demonstrated that only a low dose of OA (0.1 nM) did not affect cell morphology, even in stretched condition for 7 days and should be used in long-term experiments. The enhancement of cell growth by selective inhibition of PP2A in this experiment at least consists with the idea of growth suppression by PP2A.

Our observation of a small difference in the effect of low-dose OA treatment on cellular proliferation may be explained by the fact that PP2A is not a single enzyme but consists of different types of heterotrimers [Mayer-Jaekel and Hemmings, 1994]. OA inhibits catalytic subunit of all those various heterotrimers. Therefore, it is impossible to know which type of PP2A is playing a crucial role in cell proliferation under those experiments we performed. Most likely only a small fraction of PP2A is acting on cell proliferation and 0.1 nM OA may inhibit that fraction efficiently, but not completely. By contrast, utilization of higher concentrations of OA (1 or 10 nM) may have inhibited all of PP2A, such that many other functions of PP2A were suppressed leading to cell death.

In conclusion, cyclic strain decreased PP2A activity in the cytosol fraction of BAEC and the inhibition of PP2A by OA enhanced BAEC proliferation. Decrease of PP2A activity may play one of the key roles in enhancement of cellular growth by mechanical stretch.

REFERENCES

Begum N, Robinson LJ, Draznin B, Heidenreich KA (1993): Protein phosphatase-1 and -2a activities in cultured fetal chick neurons: Differential regulation by insulin and insulin-like growth factor-I. Endocrinology 133:2085–2090.

Bialojan C, Takai A (1988): Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Biochem J 256:283–290.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Brautigan DL, Shriner CL (1988): Methods to distinguish various types of protein phosphatase activity. Methods Enzymol 159:339–346.

Chen J, Martin BL, Brautigan DL (1992): Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. Science 257:1261–1264.

Chen J, Parsons S, Brautigan DL (1994): Tyrosine phosphorylation of protein phosphatase 2A in response to growth stimulation and v-src transformation of fibroblasts. J Biol Chem 269:7957–7962.

Cohen P, Klumpp S, Schelling DL (1989): An improved procedure for identifying and quantitating protein phosphatase in mammalian tissues. FEBS Lett 250:596–600.

Cohen P, Holmes CFB, Tsukitani Y (1992): Okadaic acid: a new probe for the study of cellular regulation. TIBS 15:98–102.

Demolle D, Lecomte M, Boeynaems J-M (1988): Pattern of protein phosphorylation in aortic endothelial cells—Modulation by adenine nucleotides and bradykinin. J Biol Chem 263:18459–18465.

van Dolah FM, Ramsdell JS (1992): Okadaic acid inhibits protein phosphatase activity in formation of the mitotic spindle of GH4 rat pituitary cells. J Cell Physiol 152:190–198.

Eriksson JE, Golman RD (1993): Protein phosphatase inhibitors alter cytoskeletal structure and cellular morphology. Adv Prot Phosphatases 7:335–357.

Ferrigno P, Langan TA, Cohen P (1993): Protein phosphatase 2A1 is the major enzyme in vertebrate cell extracts that dephospharylates several physiological substrate for cyclin-dependent kinases. Mol Cell Biol 4:669–677.

Hawker JR Jr, Granger HJ (1994): Tyrosine kinase inhibitors impair fibroblast growth factor signaling in coronary endothelial cells. Am J Physiol 266:H107–H120.

Huang X-C, Richards EM, Sumners C (1995): Angiotensin II type 2 receptor-mediated stimulation of protein phosphatase 2A in rat hypothalamic/brainstem neuronal cocultures. J Neurochem 65:2131–2137.

Ishida Y, Furukawa Y, Decaprio JA, Saito M, Griffin JD (1992): Treatment of myeloid leukemic cells with the phosphatase inhibitor okadaic acid induces cell cycle arrestt at either G1/S or G2/M depending on dose. J Cell Physiol 150:484–492.

Kim T-A, Velasquez BR, Wenner CE (1993): Okadaic acid regulation of the retinoblastoma gene product is correlated with the inhibition of growth factor-induced cell proliferation in mouse fibroblasts. Proc Natl Acad Sci USA 90:5460–5463.

Levin EG, Santell L (1991): Phosphorylation of an Mr = 29,000 protein by IL-1 Is susceptible to partial down-regulation after endothelial cell activation. J Immunol 146:3772–3778.

Lin FC, Arndt KT (1995): The role of Saccharonyces cerevisiae type 2A phosphatase in the actin cytoskeleton and in entry into mitosis. EMBO J 14:2745–2759.

Mackie K, Lai Y, Nairn AC, Greengard P, Pitt BR, Lazo JS (1986): Protein phosphorylation in cultured endothelial cells. J Cell Physiol 128:367–374.

Matsuzawa S, Matsuda A, Mizuno Y, Kikuchi K (1993): IL-2 induces transient and specific decrease in cytosolic protein phosphatase PP1 activity in murine T cell lines. Biochem Biophys Res Commun 195:989–996.

Mayer-Jaekel RE, Hemmings BA (1994): Protein phosphatase 2A-a "ménage a trois." Trends Cell Biol 4:287–291.

Mumby MC, Walter G (1993): Protein serine/threonine phosphatases: Structure, regulation, and functions in cell growth. Physiol Rev 73:673–699.

Oliveila CM, Banerjee DK (1990): Role of extracellular signaling on endothelial cell proliferation and protein N-glycosylation. J Cell Physiol 144:467–472.

Rieber M, Rieber MS (1993): Early inhibition of protein phosphatases preferentially blocks phorbol ester-stimulated mitogenic signaling in melanocytes: Increase in specific tyrosine phosphoproteins. Biochem Biophys Res Commun 192:483–491.

Rosales OR, Sumpio BE (1992): Protein kinase C is a mediator of the adaptation of vascular endothelial cells to cyclic strain in vitro. Surgery 112:459–466.

Ruediger R, Van Wart Hood JE, Mumby M, Walter G (1991): Constant expression and activity of protein phosphatase 2A in synchronized cells. Mol Cell Biol 11:4282–4285.

Sakurada K, Zheng B, Kuo JF (1992): Comparative effects of protein phosphatase inhibitors (okadaic acid and calyculin A) on human leukemia HL60, HL60/ADR and K562 cells. Biochem Biophys Res Commun 187:488–492.

Shenolikar S, Nairn AC (1991): Protein phosphatases: Recent progress. Adv Sec Messenger Phosphorylation Res 23:1–121.

Sontag E, Fedorov S, Kamibayashi C, Robbins D, Cobb M, Mumby M (1993): The interaction of SV40 small timor antigen with protein phosphatase 2A stimulates the Map kinase pathway and induces cell proliferation. Cell 75:887–897.

Sontag E, Nunbhakdi-Craig V, Bloom GS, Mumby MC (1995): A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J Cell Biol 128:1131–1144.

Sumpio BE, Banes AJ, Levin LG, Johnson G Jr (1987): Mechanical stress stimulates aortic endothelial cells to proliferate. J Vasc Surg 6:252–256.

Verin AD, Patterson CE, Day MA, Garcia JGN (1995): Regulation of endothelial cell gap formation and barrier function by myosin-associated phosphatase activities. Am J Physiol 269:L99–L108.

Walter G, Mumby M (1993): Protein serine/threonine phosphatases and cell transformation. Biochim Biophys Acta 1155:207–226.22.

Willson EW, Mai Q, Sudhir K, Weiss RH, Ives HE (1993): Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. J Cell Biol 123:741–747.

Zhou W, Takuwa N, Kumada M, Takuwa Y (1993): Protein kinase C-mediated bidirectional regulation of DNA synthesis, RB protein phosphorylation, and cyclin-dependent kinases in human vascular endothelial cells. J Biol Chem 268:23041–23048.