Suppression of Proteoglycan Synthesis by Calcium Ionophore A23187 in Cultured Vascular Endothelial Cells: Implication of Intracellular Calcium Accumulation in Lead Inhibition of Endothelial Proteoglycan Synthesis

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The purpose of the present study is to address the question of whether accumulation of intracellular calcium modulates the regulation of proteoglycan synthesis in vascular endothelial cells. Bovine aortic endothelial cells were treated with calcium ionophore A23187 in the presence of [^{35}S]sulfate, and the labeled proteoglycans were analyzed by the cetylpyridinium chloride precipitation method and DEAE-Sephacel ion-exchange chromatography. The results showed that A23187 significantly decreases the accumulation of proteoglycans, especially heparan sulfate proteoglycans, in the cell layer and the conditioned medium of vascular endothelial cells. On the other hand, it was shown that lead, a heavy metal that inhibits endothelial heparan sulfate proteoglycan synthesis, significantly increased the intracellular calcium accumulation when the accumulation was assessed by calcium-45. The present data suggest that accumulation of intracellular calcium results in a suppression of heparan sulfate proteoglycan synthesis in vascular endothelial cells; the suppression may be one of the intracellular mechanisms for lead inhibition of endothelial heparan sulfate proteoglycan synthesis.

Key words —— calcium, endothelial cell, glycosaminoglycan, heparan sulfate, proteoglycan, lead

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INTRODUCTION

Vascular endothelial cells cover the inner surface of the blood vessel wall in a monolayer. This morphologic characteristic is believed to prevent vascular lesions such as atherosclerosis by blocking direct contact between blood and the underlying tissue. When the vascular endothelium is injured, basic fibroblast growth factor (bFGF) is released from the damaged endothelial cells, and the growth factor promotes the repair of the vascular wall *via* stimulation of migration and proliferation of the cells near the damaged site in an autocrine fashion.[1)]

Proteoglycans are present in the extracellular matrix and on the cell surface in vascular endothelial cells and participate in the repair process of damaged endothelium through modulation of the activity of growth factors.[2)] For example, the binding of bFGF to the receptor is promoted by heparan sulfate proteoglycans (HSPGs) through formation of a ternary complex of bFGF, heparan sulfate chains, and the receptor.[3)]

However, the intracellular mechanism of endothelial proteoglycan metabolism has been incompletely understood. We reported that the synthesis of heparan sulfate is suppressed by cyclic AMP,[4)] and the release of glycosaminoglycans (GAGs) is promoted by activation of protein kinase C.[5)] We hypothesized that accumulation of intracellular calcium may modulate the synthesis of proteoglycans in vascular endothelial cells, since endothelial secretion of fibrinolytic proteins can be regulated by the ion.[6)]

On the other hand, lead is a heavy metal that inhibits heparan sulfate proteoglycan synthesis in vascular endothelial cells.[7)] The inhibition causes a lower response of the cells to endogenous bFGF,[8)] resulting in an inhibition of proliferation[9)] and repair[10)] after damage of the cell layers.[11)] Since lead can increase intracellular calcium levels in various cell types[12–17)] and can substitute for calcium in the intracellular signaling,[18,19)] we also hypothesized that lead inhibition of endothelial proteoglycan synthesis may partly be due to an increase in intracellular calcium. In the present study, we investigated the effect of calcium ionophore A23187 on proteoglycan synthesis and that of lead on the accumulation of intracellular calcium in cultured vascular endothelial cells.

MATERIALS AND METHODS

Materials —— Vascular endothelial cells derived from bovine aorta were purchased from Dainihon Pharmaceutical (Osaka, Japan). RPMI 1640 medium and ASF 301 medium were from Nissui Pharmaceutical (Tokyo, Japan) and Ajinomoto (Tokyo, Japan), respectively. Fetal bovine serum (FBS) was obtained from Summit Biotechnology (Fort Collins, CO, U.S.A.). Tissue culture dishes and plates were from Corning (NY, U.S.A.). $[^{35}S]Na_2SO_4$ (carrier free) and calcium-45 as $[^{45}Ca]Cl_2$ (865.8 MBq/mg) were purchased from New England Nuclear (Boston, MA, U.S.A.). The PD-10 column (disposable Sephadex G-25M) was from Amersham Pharmacia Biotech (Amersham, U.K.). DEAE-Sephacel, benzamidine, and phenylmethanesulfonyl fluoride were obtained from Sigma (St. Louis, MO, U.S.A.). A commercially available lactate dehydrogenase assay kit and calcium ionophore A23187 were from Wako Pure Chemical Industries (Osaka, Japan). Cetylpyridinium chloride (CPC), lead nitrate, and other reagents were from Nacalai Tesque (Kyoto, Japan).

Accumulation of $[^{35}S]$Sulfate-labeled GAGs —— Vascular endothelial cells were cultured in RPMI-1640 medium supplemented with 10% FBS in 100-mm dishes at 37°C in a humid atmosphere of 5% CO_2 in air until confluent. They were then transferred into 24-well culture plates at 1×10^4 cells/cm^2 and cultured for 24 hr in RPMI-1640 medium supplemented with 10% FBS. The sparse cultures of vascular endothelial cells were incubated at 37°C for 6, 12, 24 or 48 hr in 0.25 ml of serum-free ASF 301 medium with A23187 (0.05, 0.1, or 0.2 μM) in the presence of $[^{35}S]$sulfate (370 kBq/ml). The incorporation of $[^{35}S]$sulfate radioactivity into GAGs accumulated in the cell layer and the conditioned medium was determined by the CPC precipitation method[20] as described previously.[21]

DEAE-Sephacel Ion-exchange Chromatography —— Sparse cultures of vascular endothelial cells were prepared in 60-mm dishes and incubated at 37°C for 24 hr in 2 ml of serum-free ASF 301 medium with A23187 (0.1 μM) in the presence of $[^{35}S]$sulfate (740 kBq/ml). The conditioned medium was harvested and solid urea was added to a concentration of 8 M (the medium extract). The cell layer was washed twice with ice-cold Ca- and Mg-free phosphate-buffered saline (CMF-PBS) and extracted with 8 M urea solution containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 10 mM *N*-ethylmaleimide, 2 mM EDTA, 0.1 M phenylmethanesulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100 (pH 7.5) at 4°C for 15 min; the cell layer was harvested by scraping with a rubber policeman (the cell extract). The medium and cell extracts were chromatographed on PD-10 columns equilibrated in 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.1 M NaCl, 0.5% Triton X-100, and 50 mM Tris base to remove low molecular-mass (> 3 kDa) materials. To separate proteoglycans into HSPGs and chondroitin/dermatan sulfate proteoglycans (CS/DSPGs) on the basis of differences in charge density, the macromolecules were applied to DEAE-Sephacel (5 ml of resin) in the urea buffer, and unbound radioactivity was removed from the column by washing with 30 ml of the buffer. Bound radioactivity was eluted from the column with a liner gradient of 0.1–0.7 M NaCl in the urea buffer (total volume of 50 ml).

Intracellular Accumulation of Calcium-45 —— Sparse cultures of vascular endothelial cells were prepared in a 24-well culture plate and incubated at 37°C for 24 hr in 0.25 ml of fresh serum-free medium with lead nitrate (0.5, 1, 2, or 5 μM) in the presence of calcium-45 (370 kBq/ml). After incubation, the medium was discarded and the cell layer was washed three times with CMF-PBS containing 2 mM EGTA at 4°C to remove calcium ions loosely bound to the cell surface.[22] The cell lysate was prepared by the addition of 0.25 ml of 0.5% SDS to the cells. After collection of the cell lysate, the culture well was washed with 0.25 ml of CMF-PBS and the wash was combined with the cell lysate. The radioactivity in the cell lysate was measured by liquid scintillation counting. Separately, sparse cultures were treated with lead in the same conditions in the absence of calcium-45, and the DNA content was determined by fluorometric methods.[23] The accumulation of calcium-45 radioactivity in the cells was expressed as dpm/μg DNA.

Statistical Analysis —— Data on the incorporation of $[^{35}S]$sulfate into GAGs and the accumulation of calcium-45 were analyzed for statistical significance using ANOVA and Bonferroni's multiple *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Figure 1 shows the accumulation of proteoglycans in which GAG chains were labeled

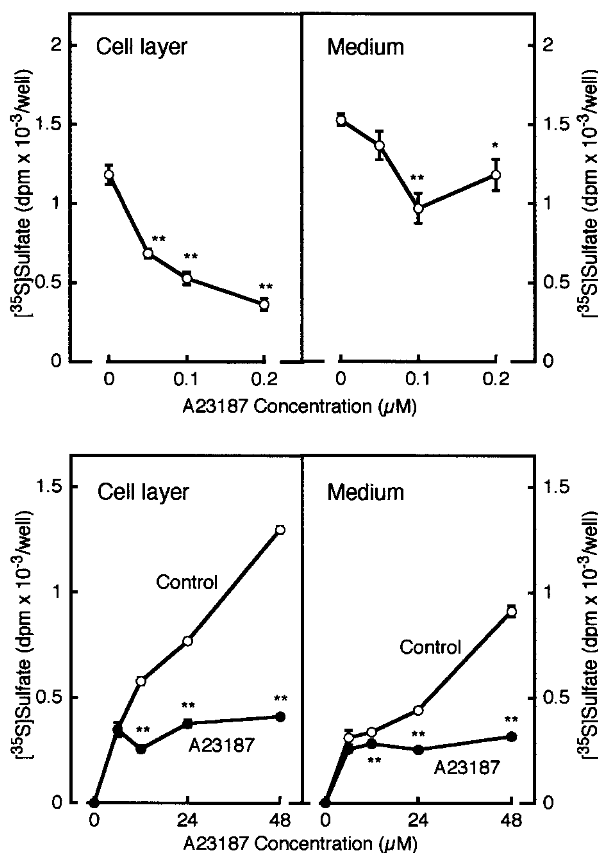

Fig. 1. Accumulation of [^{35}S]Sulfate-labeled Proteoglycans in the Cell Layer and the Conditioned Medium of Vascular Endothelial Cells Treated with Calcium Ionophore A23187

Sparse cultures of bovine aortic endothelial cells were incubated at 37°C for 24 hr with A23187 at 0.5, 0.1, or 0.2 μM (upper panels) or for 6, 12, 24, or 48 hr with A23187 at 0.1 μM (lower panels) in the presence of [^{35}S]sulfate at 370 kBq/ml. Values are means ± S.E. of four samples. Significantly different from the corresponding control, $*p < 0.05$; $**p < 0.01$.

with [^{35}S]sulfate in the cell layer and the conditioned medium of vascular endothelial cells after treatment with calcium ionophore A23187. The ionophore significantly decreased the [^{35}S]sulfate-labeled proteoglycans in the cell layer at a concentration of 0.05 μM and more and in the conditioned medium at 0.1 μM and more after 24-hr incubation. When the cells were treated with A23187 0.1 μM, the decrease in the proteoglycan accumulation by the ionophore was observed after 12 hr and longer in both the cell layer and the conditioned medium. The leakage of cytoplasmic lactate dehydrogenase, a marker of nonspecific cell damage, from the cells into the medium, was unchanged by A23187 after 24-hr treatment (control, 8.90 ± 0.78 IU/l; A23187 0.05 μM, 8.36 ± 0.95 IU/l; A23187 0.1 μM, 9.41 ± 0.92 IU/l; A23187 0.2 μM, 11.69 ± 0.68 IU/l; $n = 4$). These results indicate that the accumulation of intracellular calcium results in suppression of proteoglycan synthesis in vascular endothelial cells.

Figure 2 shows the DEAE-Sephacel profiles of [^{35}S]sulfate-labeled proteoglycans extracted from the cell layer and the conditioned medium of vascular endothelial cells treated with or without A23187. Two peaks of [^{35}S]sulfate radioactivity were eluted from the column with approximately 0.45 and 0.55 M NaCl; these two peaks have been previously confirmed to contain HSPGs and CS/DSPGs,[24] respectively. It was shown that A23187 markedly decreased the radioactivity of HSPGs in the cell layer and the conditioned medium (55% and 18%, respectively, of the control value). CS/DSPGs were also decreased by the ionophore in both the cell layer and the conditioned medium (47% and 48%, respectively, of the control value). These results indicate that the synthesis of HSPGs rather than CS/DSPGs is preferentially suppressed by intracellular accumulation of calcium in vascular endothelial cells.

Figure 3 shows the intracellular accumulation of radioactive calcium in vascular endothelial cells after 24-hr treatment with lead. Lead at 2 μM or more significantly increased the calcium-45 accumulation, suggesting that the metal promotes the accumulation of calcium within the cells.

DISCUSSION

Intracellular calcium is a key second messenger that mediates the stimulation of chemical, physical, and electrical stimulation as well as the regulation by physiologic factors. It has been shown that the regulation of proteoglycan synthesis can also be mediated by intracellular calcium in several cell types other than vascular endothelial cells. Calcium antagonists such as calcium channel blockers inhibit proteoglycan synthesis in vascular smooth muscle cells,[25] Sertoli cells,[26] granulosa cells,[27] and chondrocytes,[28] suggesting that an increase in intracellular calcium promotes proteoglycan synthesis in these cell types. In contrast, interleukin-1 inhibits proteoglycan synthesis in chondrocytes *via* an increase in intracellular calcium,[29] indicating that calcium signaling may mediate the suppression of proteoglycan synthesis in this cell type. In the present study, it was shown that calcium ionophore A23187 suppressed the synthesis of proteoglycans, especially HSPGs, in vascular endothelial cells. Taken together, these results suggest that regulation of proteoglycan

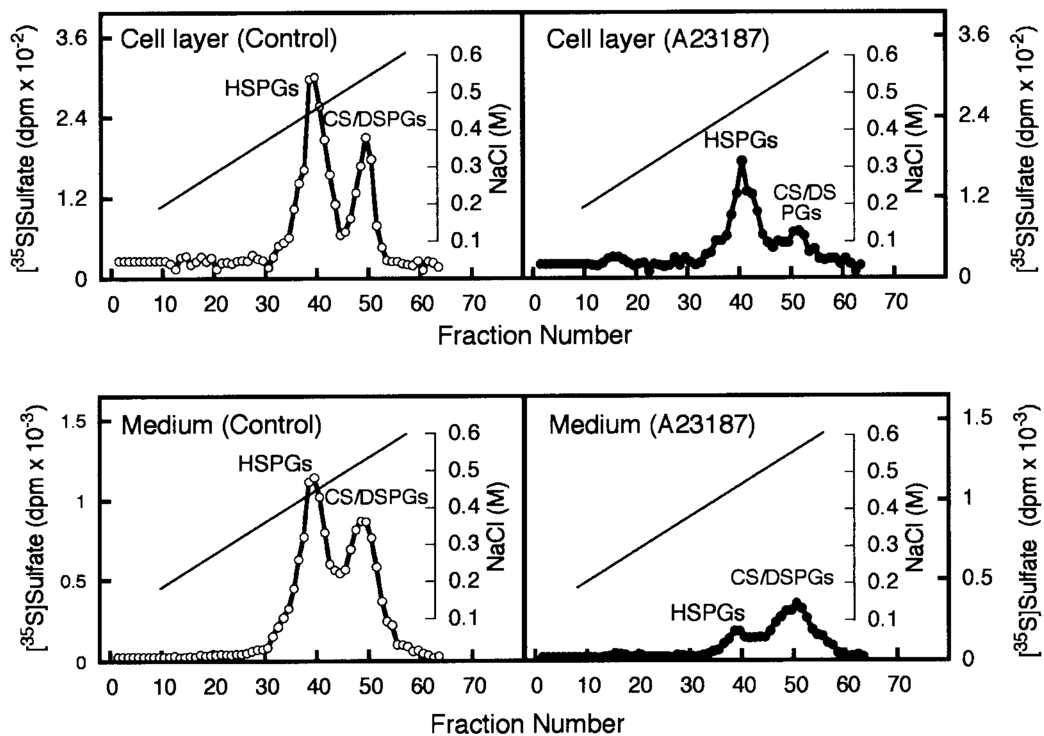

Fig. 2. DEAE-Sephacel Ion-exchange Chromatography of [^{35}S]Sulfate-labeled Proteoglycans Extracted from the Cell Layer and the Conditioned Medium of Vascular Endothelial Cells Treated with Calcium Ionophore A23187 with a Linear Gradient of 0.1 to 0.7 M NaCl in 8 M Urea Buffer

Sparse cultures of bovine aortic endothelial cells were incubated at 37°C for 24 hr with A23187 at 0.1 μM in the presence of [^{35}S]sulfate at 740 kBq/ml.

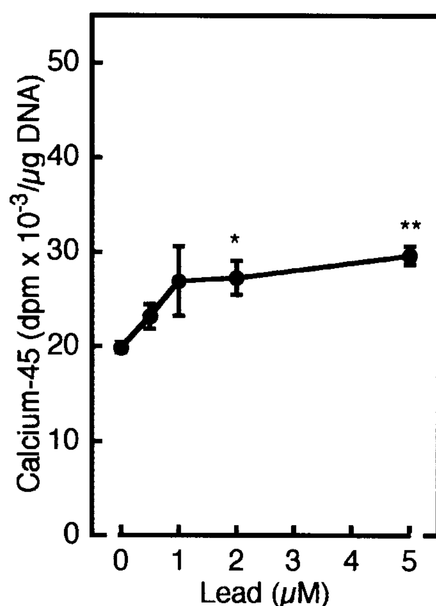

Fig. 3. Intracellular Accumulation of Calcium-45 in Vascular Endothelial Cells Treated with Lead

Sparse cultures of bovine aortic endothelial cells were incubated at 37°C for 24 hr with lead nitrate at 0.5, 1, 2, or 5 μM in the presence of calcium-45 at 370 kBq/ml. Values are means ± S.E. of four samples. Significantly different from the control, *$p < 0.05$; **$p < 0.01$.

synthesis is mediated by intracellular calcium in a cell type-dependent manner, and that vascular endothelial cells are a cell type in which proteoglycan synthesis is suppressed by intracellular calcium accumulation.

The proliferation of vascular endothelial cells is inhibited but that of vascular smooth muscle cells is stimulated by the heavy metal lead.[9,30] Calcium ionophore A23187 also modulates the proliferation of vascular endothelial and smooth muscle cells in a similar manner to that of lead.[30] Lead characteristically perturbs the calcium-dependent intracellular regulation pathway by increasing the intracellular calcium level and/or mimicking calcium in various cell types,[12–17] although the metal can be an effective antagonist of calcium transients under certain conditions.[31–33] In the present study, it was shown that A23187 suppresses the synthesis of proteoglycans, and lead increases the accumulation of intracellular calcium, suggesting that lead inhibition of endothelial proteoglycan synthesis[7,8] is mediated by the intracellular calcium increased by the metal. On the other hand, A23187 stimulates the secretion of tissue plasminogen activator but sup-

presses that of plasminogen activator inhibitor type 1,[6] whereas lead inhibits the secretion of the activator but does not influence that of the inhibitor;[34] this effect of lead was suggested to be mediated by the cyclic AMP-dependent pathway.[34] Thus it is suggested that the intracellular mechanism that participates in lead toxicity in vascular endothelial cells includes not only the calcium-dependent pathway but also other pathways such as the cyclic AMP-dependent pathway. It is a moot point whether or not intracellular mechanisms other than the calcium-dependent pathway contribute to lead inhibition of endothelial proteoglycan synthesis.

In summary, the present data suggest that an increase in intracellular calcium results in suppression of proteoglycan synthesis in vascular endothelial cells. Since lead increased the calcium accumulation within the cells, the suppression may be involved in the lead inhibition of endothelial proteoglycan synthesis. Calcium antagonists show antiatherogenic properties,[35–37] suggesting the possibility that an increase in intracellular calcium in vascular endothelial cells results in progression of atherosclerosis. Since endothelial proteoglycans not only promote the repair of damaged endothelium[1–3] but also exhibit anticoagulant activity on the inner surface of blood vessel wall,[38,39] it is suggested that excess accumulation of intracellular calcium may induce a retardation of endothelial repair by inhibition of endothelial cell proliferation[9,10] and a procoagulant state of blood on the cells by inhibition of endothelial proteoglycan synthesis. Lead-induced increases in intracellular calcium in vascular endothelial cells may cause similar changes in the endothelium and promote the progression of atherosclerosis.[40–42]

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