Regulatory effect of thromboxane on proliferation of vascular smooth muscle cells from rats

ラット血管平滑筋細胞増殖に及ぼすトロンボキサンの影響

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REGULATORY EFFECT OF THROMBOXANE ON PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELLS FROM RATS

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SUMMARY

We investigated the regulatory effects of the vasoconstrictor, thromboxane, on the proliferation of vascular smooth muscle cells (VSMC) from Wistar-Kyoto rats (WKY), using 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2), a stable analogue of thromboxane A_2. STA_2 increased incorporation of (^{3}H)thymidine into deoxynucleic acid (DNA) in randomly cycling VSMC in a dose-dependent manner, and this compound significantly shortened the doubling time of VSMC. Cell cycle analysis revealed that the increase in VSMC growth was primarily due to a rapid transition from the DNA synthetic (S) to the G2/mitotic (M) phase. Moreover, STA_2 enhanced protein synthesis in VSMC during the G2/M phase, whereas protein synthesis was unaffected in the GO/G1 period. In fact, sodium laurylsulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) analysis indicated that STA_2 prompted the cells in G2/M phase to synthesize actin, a major cytoskeleton protein. Conversely, inhibition of protein synthesis by puromycin retarded the transition from the S to the G2/M period. In addition, depolymerization of the actin molecules by cytochalasin D offset the quick progression to the G2/M phase by STA_2. Thus, these data indicate that thromboxane stimulates the growth of randomly cycling VSMC and that this is primarily due to a rapid transition from S to G2/M. This enhanced progression is attributable in part to a rapid buildup of cytoskeleton proteins during the G2/M period.

INTRODUCTION

Recent studies have emphasized the role of the growth and migration of medial smooth muscle cells in the remodeling of the vascular structure in a hypertensive state (1,2,3). It is therefore of great interest to investigate the regulatory mechanism of the proliferation of vascular smooth muscle cells (VSMC). A number of these studies have thus far been conducted, thereby providing evidence for the involvement of various vasoactive substances derived from vascular vessels in the modulation of VSMC growth (4,5,6,7).

The vascular wall is not only the container for the blood supply, but also functions as the active site in the production of various vasoconstrictor and relaxing substances, e.g. eicosanoids, kinins, endothelium-derived relaxing factor(s), endothelin and some growth factors (8,9). These vasoactive substances additively or synergistically contribute, as paracrine or autocrine hormones, to the regulation of vascular wall function. Moreover, recent studies indicate that these substances, at least in part, influence the proliferation of smooth muscle cells in the arterial vessels (4,5,6,7).

In fact, it has been reported that vasodepressor prostaglandins, e.g. prostacyclin (PGI_2), prostaglandin E_2 (PGE_2) and PGD_2, retard the proliferation of VSMC in vitro (4,10). Conversely, the vasoconstrictor, thromboxane, enhances VSMC proliferation (11). The vascular wall has a great capacity for eicosanoid generation. In experimental models for hypertension, the vascular eicosanoid system is altered even during the prehypertensive state (12,13). Based on these

studies, it seems possible that the vascular eicosanoid system plays some role in the remodeling of the vascular structure in a hypertensive state.

Although this hypothesis is intriguing, the possible involvements of thromboxane in the proliferation of VSMC has not been fully addressed. We therefore have investigated whether and the mechanism by which the vasoconstrictor, thromboxane, influences the proliferation of smooth muscle cells derived from rat thoracic aorta, using STA_2, a stable analogue of thromboxane A_2, and cell culture techniques.

MATERIALS AND METHODS

Cell Culture

VSMC were isolated from the thoracic aortas of 8-week-old Wistar-Kyoto rats (WKY), according to the method of Ross (14,15). Briefly, thoracic descending aortas were obtained, and the adventitia and intima were carefully removed. Then, the explants were placed on a polystyrene dish with the intimal side attached to the dish, and Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, New York, USA), containing 20% (v/v) fetal bovine serum (FBS; Gibco Laboratories), penicillin (100 U/ml), and streptomycin (100 μg/ml), was gently added. The dishes were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells reached confluence 10 days after inoculation. They were harvested by brief exposure to Hanks' solution supplemented with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA, and transferred into a new dish. At this stage, the cells were designated as the first passage. The percentage of FBS was changed to 10% (v/v) in subsequent media. The harvest was repeated when the cells became confluent. VSMC from passages 3 to 6 were used in this study. The cells in culture exhibited the characteristic "hills and valleys" growth pattern, and the presence of alpha-actin molecules was demonstrated by using a monoclonal anti-alpha-actin antibody and immuno-enzyme assay method (4,15).

Cell Synchrony

Cell synchronization was performed according to the standard methods as described by Ashihara (16). In order to synchronize VSMC in the G0/G1 boundary (quiescent VSMC), randomly cycling cells were

cultured in serum-free media for 48 hours. Replacement of serum-free media by 10% fresh sera initiated cell proliferation.

Quiescent VSMC were cultured for 24 hours in media containing 10% sera and 1mM hydroxyurea. Hydroxyurea stopped the proliferative process at the G1/S boundary. DNA replication was started again by washout of the hydroxyurea with 10% fresh sera.

In order to synchronize the cells in the premitotic period, we utilized 15 ng/ml colchicine. G1/S-synchronized VSMC were cultured for 12 hours in DMEM containing 10% sera and colchicine. The mitotic process could be resumed by washout of the colchicine with 10% fresh sera.

Assessment of Cell Proliferation

(1)Doubling Time

To assess the proliferating activity, we measured the doubling time of VSMC and (^{3}H)thymidine uptake into DNA fragments. The doubling time was determined as follows. $5x10^4$ cells were seeded onto 9-cm^2 dishes with 2ml of DMEM containing 10% sera and cultured for 24 hours. Then the media were changed to culture media containing a given concentration of STA_2 (9,11-epithio-11,12-methano-thromboxane A_2, Ono Pharamceutical Co., Ltd., Kyoto, Japan), a stable thromboxane A_2 analogue. Cell number was determined by light microscopy before the addition of STA_2 and after 24 hours. The doubling time was calculated according to the following equation: doubling time = $(t_1 - t_2)x\log 2/(\log N_1 - \log N_2)$. In this equation, t_1 and t_2 are the times when cell numbers are counted and N_1 and N_2 are the cell numbers at t_1 and t_2, respectively.

(2) Measurement of (^{3}H)Thymidine Uptake

The rate of DNA replication was assessed by measuring incorporation of (^{3}H)thymidine into DNA fragments (4). First, VSMC in a logarithmic growth state were cultured for 24 hours in media containing 10% FBS, 0.25 μCi of (^{3}H)thymidine (20-30 Ci/mmol) and a given concentration of STA_2. After the culture period, the cells were harvested, washed with Dulbecco's phosphate buffered saline (D-PBS) without Ca^{2+} and Mg^{2+} and then homogenized by a model UR-20 ultrasonicator (Tomy-Seiko Co., Ltd., Tokyo Japan). The homogenate was treated with 5% perchloric acid at 4°C for 30 minutes and spun at 3000g for 30 minutes. The precipitate was repeatedly washed with 5% perchloric acid solution and radioactivity in this acid-insoluble fraction was measured by an automatic liquid scintillation counter.

Similarly, (^{3}H)thymidine uptake by GO/G1-synchronized VSMC or G1/S-synchronized VSMC was measured. In these experiments, the cells were cultured in fresh media containing (^{3}H)thymidine and 10^{-5}M STA_2 and harvested at various culture periods. Radioactivity in the acid-insoluble DNA fragments was determined by the same procedures as described in the method for randomly cycling VSMC.

(3)Mitotic Index

The per cent population of mitotic cells (mitotic index) was determined by light microscopy. Briefly, after the proliferating process was resumed in the VSMC synchronized in various phases, the cells were fixed with 5% acetate ethanol solution after culture for various time periods. The fixed cells were stained by Giemsa solution. The cells in the mitotic process were detected by light

microscopy. Five hundred nuclei were examined in each sample.

Cytoskeleton Protein Synthesis

To examine STA_2 effect on cell body formation, we determined incorporation of (^{3}H)leucine into cellular proteins. VSMC synchronized in the G0/G1 or G1/S boundary were cultured for 12 hours in 10% FBS containing a given concentration of STA_2 and 0.05 μCi of (^{3}H)leucine (120-190 Ci/mmol). The cells were harvested and homogenized by an ultrasonicator in 25mM phosphate-buffered saline containing 1mM EDTA. The homogenate was treated with ice-chilled 5% (v/v) trichloracetate. The precipitate was washed with the trichloracetate solution and dissolved in 1 ml of 0.5 N NaOH solution. The radioactivity of this solution was measured with an automatic liquid scintillation counter.

To reveal whether thromboxane directly affected the synthesis of cytoskeleton proteins during the G2/M period, we then examined the alterations in the formation of actin, a major cyctoskeleton protein, using sodium laurylsulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) (17). VSMC in culture were harvested and homogenized by an ultrasonicator. The homogenate was spun at 10^4g for 30 minutes. The pellet was washed three times with 5 mM phosphate-buffered solution at pH 7.2 including 1mM ethylenediamine-tetraacetate disodium (EDTA) and 1mM dithiothreitol. The supernatant was combined and used for actin analysis. Briefly, the proteins in the supernatant were denatured at 95 °C for 10 minutes in 50 mM Tris-Cl at pH 6.8 containing 10% (v/v) glycerol, 2% (w/v) SDS, 1 mM dithiothreitol and 0.0025% (w/v) bromophenol blue. Samples were applied to a 7.5% polyacrylamide slab gel, using a PROTEAN II Slab Cell (Bio-Rad, Richmond, California,

U.S.A.) and MultiDrive XL power supply unit (Pharmacia LKB Biotechnology, Bromma, Sweden). Proteins were stained with 0.1% Coomassie blue in fixative (water:methanol:acetate=5:4:1, v/v) and destained. To compare the actin in VSMC with that in the aortic vascular wall, we excised thoracic aorta from WKY and prepared 10^4g supernatant. The SDS-PAGE analysis was performed in the same procedures as described in this section. Figure 1 shows a representative separation pattern of the SDS-PAGE analysis. An apparent band was found at a relative mobility of 42kD, which corresponds to the molecular weight of the standard actin (Sigma Chemical Co., St.louis, MD, USA). Diluted homogenates were quantitatively assessed, using a model EPA-3000 density-pattern analyzer (Maruzen Petrochemical Co., Ltd., Tokyo, Japan). Actin densities in the diluted homogenates well paralleled the dilution titers (r=0.99) (Figure 2).

G1/S-synchronized cells were cultured for 12 hours in media containing 0.05 μCi (^{3}H)leucine and a given concentration of STA_2. Harvested VSMC were treated as mentioned above, and applied to SDS-PAGE. The density of the actin band was scanned as above. Moreover, alterations in actin formation were determined by measuring incorporation of (^{3}H)leucine into actin molecules. For this analysis, the actin band was cut out of the gel and the radioactivity in it was eluted with a tissue solubilizer (NCS, Amersham International, Buckinghamshire, England). The radioactivity was determined using an automatic liquid scintillation counter.

In order to confirm the association of cytoskeleton protein formation with cell growth, we investigated the effects of protein-

synthesis inhibition and functional disturbance of actin on the mitotic process in the M phase. G1/S-synchronized VSMC were cultured in fresh media containing a given concentration of puromycin, an inhibitor of protein synthesis, or cytochalasin D, a depolymerizing agent of actin. The mitotic index was measured at various culture periods.

Reagents

Reagents were all of analytical grade. Eicosanoids and related compounds were offered by Ono Pharmaceutical Co., Ltd., Osaka, Japan. Radioactive materials were purchased from Amersham International, Buckinghamshire, England.

Statistical Analysis

The values are expressed as means $\pm$ SE. Statistical differences were analyzed by the Student's t test, the paired t test, or one or two way analysis of variance.

RESULTS

Thromboxane Effect on Randomly Cycling VSMC

First, we investigated the effect of thromboxane on the cell growth of randomly cycling VSMC. STA_2, a stable analogue of the vasoconstrictor, thromboxane, increased incorporation of (^{3}H)thymidine into acid-insoluble DNA fragments in VSMC in a dose-dependent manner ranging from 10^{-8} to 10^{-4}M STA_2 (Table 1). To confirm this mitogenic effect of thromboxane, we examined the alteration in the doubling time when the cells were maximally stimulated with 10^{-5}M STA_2. As shown in Figure 3, this dose of STA_2 significantly shortened the doubling time of VSMC in a logarithmic growth state (19.24 $\pm$ 0.32 vs 20.25 $\pm$ 0.40 hours). These data clearly indicate that STA_2, a stable analogue of thromboxane, enhances the growth of randomly cycling VSMC.

Analysis of Enhanced Cell Proliferation

To investigate the mechanism of the enhanced cell growth by STA_2, we examined each period in the proliferating cell cycle, using cell synchrony techniques. Quiescent VSMC were stimulated with 10% sera at zero time (Figure 4). The cells initiated incorporation of (^{3}H)thymidine into acid-insoluble DNA fragments after approximately 12 hours, indicating the initiation of the S period. 10^{-5}M STA_2, the dose of which enhances the VSMC growth, did not alter the initiation time of DNA replication. In contrast, in the presence of 10^{-5}M STA_2, the mitotic curve was shifted toward left, indicating that a shortening of the transition from the G0/G1 to the M period. These data strongly suggest that the transition from the G0/G1 to the S period

is not influenced, but the transition from the S to the M period is significantly shortened, by STA_2.

To clarify this difference, we examined alterations in the progression, using VSMC synchronized in the G1/S boundary. The cycling process was stopped at this boundary with hydroxyurea and started again by washout of this reagent with fresh media at zero time (Figure 5a). DNA replication was initiated immediately after the washout, as indicated by an increase in (^{3}H)thymidine uptake. The uptake was time-dependently increased both in STA_2-free media and in media containing 10^{-5}M STA_2. There was no difference in the replication rate between the two groups. In contrast, the mitotic index curve was significantly shifted toward the left when the cells were stimulated with 10^{-5}M STA_2. To assess the specificity of STA_2 to the premitotic and mitotic processes, we examined whether 10^{-2} U/ml PDGF (Transformation Research INC, Framingham, MA, USA), being a potent competence factor, affects this mitotic process. This dose of PDGF is known to be equivalent to 10% FBS in the mitogenic activity. As shown in Figure 5b, PDGF did not influence the transition from the G1/S to the M period. These data support the proposal that thromboxane does not affect DNA replication, but instead, specifically facilitates the transition from the S to the G2/M phase.

Furthermore, we attempted to investigate the effects of thromboxane on cell division per se, using the cells synchronized in a premitotic period. The mitotic process was resumed by washout of colchicine at zero time (Figure 6). As shown in the figure, 10^{-5}M STA_2 caused the cells to enter the mitotic process. Moreover, it should be

noted that a 3-hour incubation time was required to make the effect of thromboxane obvious.

To assess the dose-response relationship between STA_2 and the enhanced mitotic process, we measured the mitotic index at 8 hours after washout of hydroxyurea when the cells were cultured in media containing a given concentration of STA_2. As shown in Table 2, STA_2 increased the mitotic index in a dose-dependent manner ranging from 10^{-8} to 10^{-5}M.

Cytoskeleton Protein Synthesis and Thromboxane

To reveal the mechanism of the thromboxane effect on the progression in the G2/M period, we focused on the STA_2 effect on the protein synthesis required to form the daughter cells, because protein synthesis is a major event during the G2 and the M periods. First we measured incorporation of (^{3}H)leucine into the acid-insoluble protein fraction of VSMC. The left graph of Figure 7 illustrates the amounts of (^{3}H)leucine incorporated into this fraction during the GO/G1 period. STA_2 did not influence the incorporation at the concentrations ranging from 10^{-9} to 10^{-5}M. In contrast, as shown in the right graph of Figure 7, protein synthesis was significantly enhanced by STA_2 when the cells were in the G2 or M period.

To define whether STA_2, in fact, stimulates the formation of the cell body, we measured the effect of STA_2 on the synthesis of actin, a representative cytoskeleton protein, in dividing cells. The left graph of Figure 8 shows the densitometric analysis of the actin component in the cell homogenate and the right, the radioactivity of (^{3}H)leucine incorporated into actin molecules. Both results, using

different assay methods, clearly indicate that STA_2 enhances the formation of actin during in the G2/M period.

Finally, we investigated the relationship between the formation of cytoskeleton proteins and the progression of the mitotic process. When protein synthesis in the dividing cells was inhibited by puromycin, a protein synthesis inhibitor, the progression of the mitotic process was significantly retarded (Figure 9). Similarly, when the function of actin was disturbed by 10^{-7}M cytochalasin D, a depolymerizing reagent specific for actin molecules, the enhanced transition from the S to the G2/M period caused by thromboxane was eliminated (Figure 10).

DISCUSSION

In this study, we clearly demonstrated that thromboxane significantly enhanced the formation of DNA in randomly cycling VSMC. The mitogenic effects of STA_2 were also supported by the finding that doubling time of the VSMC became shortened when the proliferative cells were cultured in media containing 10^{-5}M STA_2. These results are in accordance with those of previous studies (4,11).

To analyze the mitogenic property of STA_2, we investigated the alterations in each process of the cell proliferation, using cell synchronization techniques. It was demonstrated that 10^{-5}M STA_2, the dose of which stimulates the proliferation of VSMC in a logarithmic growth state, did not affect the transition from G0/G1 to S phase. Moreover, this dose of thromboxane did not influence DNA replication during the S phase. However, when the cells were in the G2 resting or mitotic period, the process of cell proliferation was significantly enhanced in media containing 10^{-5}M STA_2. Thus, these data clearly indicate that the rapid proliferation of VSMC induced by STA_2 is primarily due to the quick transition from the S to the G2/M period.

This proposal is supported by the results from the G1/S-synchronized VSMC. DNA replication was not influenced by thromboxane; however, the mitotic process was prematurely initiated when the cells were cultured with STA_2. More directly, the study of M-synchronized VSMC provided evidence for the stimulation of the mitotic process by the thromboxane.

The effect on the G2/M period is unique to vasoconstrictor thromboxanes because vasodepressor prostaglandins primarily prolong

transition from the G0/G1 to the S period (18). In fact, in neoplastic cell lines, it has been found that vasodilator prostaglandins arrest the cells at the G1 period (19). During G1 period, various oncogenes are expressed and the cells are set up for subsequent DNA replication (20). In addition, the cells are promoted to synthesize cytoskeleton proteins for duplication of the cell body. In the present study, we found that thromboxane stimulates protein synthesis by the cells in G2 resting and mitotic periods, but not during the G0/G1 period. The formation of actin, a representative cytoskeleton protein, was significantly increased by STA_2. Thus, it seems probable that the thromboxane makes the cells prepare for cell duplication through stimulating synthesis of cellular proteins.

The mechanism of the thromboxane effect on protein synthesis is unclear. However, there is some evidence that thromboxane increases intracellular free calcium concentration (21,22). In this context, we have found that deprivation of the extracellular calcium by a calcium chelating reagent, EDTA, terminates the mitotic process and that a calcium channel blocker retards progression of mitosis as well (23). Although we did not have direct evidence of the involvement of the cytosolic free-calcium level, an increase in intracellular calcium by thromboxane could be a cause of the enhanced synthesis of cytoskeleton proteins. However, it is obvious that we cannot address these points conclusively until we measure the time-dependent alterations in the intracellular calcium concentration in proliferative VSMC.

There is much evidence that the thromboxane system is enhanced in the vascular wall and kidney in genetic rat models for human hy-

pertension (24,25). The enhancement of the thromboxane system precedes the development of hypertension (24,25). Thus, it is of great interest to investigate the implications of this enhanced system in the genesis of hypertension. However, the relationship has not been fully proven thus far. In this context, the fact that thromboxane enhances VSMC growth may provide evidence for the importance of the thromboxane system in the medial hypertrophy in the vascular wall, an integral component of genetic hypertension. Indeed, there is evidence in vitro that endogenous thromboxanes participate in the quick proliferation of VSMC from spontaneously hypertensive rats (11). Inhibition of endogenous thromboxane generation by OKY-046, a thromboxane synthase specific inhibitor, retards the enhanced proliferation of VSMC from spontaneously hypertensive rats.

There are some debates as to whether the medial hypertrophy is due to hyperplasia or hypertrophy of VSMC. The mechanism is dependent on the size of arteries (26,27,28). In the present study, we demonstrated that thromboxane stimulates cytoskeleton protein synthesis, particularly during the G2/M period, and this increment was associated with a quick progression of the mitotic process. In fact, there is some evidence suggesting that actin molecules are involved in the progression of cell proliferation. Cytochalasin B and phalloidin, inhibitors of actin polymerization, inhibit the cell duplication process in eukaryotic cells (29,30). Moreover, temperature-sensitive mutation was created in yeast cells, the mutant actin of which functions normally at low (15 °C) temperature, thereby prompting the cells to grow and proliferate, whereas at elevated

temperatures, the actin filaments were disrupted and the cells were unable to divide (31). These data indicate that there is a close relationship between the cytoskeletal protein, actin, and the progression of cell proliferation. To address these points more clearly, anti-actin specific antibody has to be raised and the influence of this antibody in the cell proliferation has to be investigated.

In the present study, we examined STA_2 effects under experimental conditions in which endogenous eicosanoid generation was not inhibited by indomethacin. This was done in an attempt to avoid the additional effects of nonsteroidal antiinflammatory agents on VSMC growth, which are independent of the inhibition of eicosanoid generation. Moreover, the medium was not supplemented with exogenous arachidonate so that this could minimize the endogenous eicosanoid generation (10). These procedures were of use to investigate the actions of the exogenous thromboxane without influences of the endogenous eicosanoid system.

In conclusion, we analyzed the mechanisms of the thromboxane effects on randomly cycling VSMC, using cell synchronization techniques. Although there may be some difference between the randomly cycling VSMC and synchronized cells, we have proposed that the shortening of G2/M period is the mechanism of responsible for rapid proliferation in randomly cycling VSMC. Moreover, the time-difference in proliferation of randomly cycling VSMC was about one hour, and this difference was similar to the time-difference seen in G1/S-synchronized VSMC. These data indicate that even if other mechanisms

are invovled in the quick proliferation by thromboxane, the predominant mechanism appears to be due to the alterations in the G2/M phase. Thus, thromboxane could play a pathophysiological role in the stimulation of vascular smooth muscle cell proliferation, and thereby, contribute to the hypertrophy of the arterial wall seen in some hypertensive conditions.

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FIGURE LEGENDS

Figure 1. Analysis of Cytoskeleton Proteins by SDS-PAGE

The homogenates of cultured VSMC and aortic walls were analyzed by SDS-PAGE, as described in text. Actin molecules were identified at 42 kD, refering to the molecular weight standards (the left column, Bio-Rad Cat#161-0303). 200kD = myosin, 116kD = E.coli beta-galactosidase, 97kD = rabbit muscle phosphorylase b, 66kD = bovine serum albumin, 43kD = hen egg white ovalbumin.

Figure 2. Validity of Quantitative Densitometry of Actin

Actin molecules in the diluted 10^4g supernatant were analyzed by SDS-PAGE and densitometric assay method as described in text. Rm represents the relative mobility of each peak. OD_{517}xdRm represents the integrated absorbance of the separated band.

FIGURE 3. Effects of STA_2 on the Doubling Time

10^4 cells were inoculated and cultured for 48 hours. Then, doubling time of the cells in a logarithmic growth state was measured according to the method described in text. Open circles represent the doubling times of the cells cultured in STA_2-free media and closed circles those in media containing 10^{-5}M STA_2. The difference was assessed by the paired t test.

FIGURE 4. Analysis of the Proliferative Process in Quiescent VSMC

10^4 cells were inoculated and cultured for 48 hours. Then, the

cells were synchronized in the G0/G1 boundary by deprivation of sera in media. Proliferation of the quiescent VSMC was stimulated by media containing 10% serum at zero time. (^{3}H)Thymidine uptake (broken lines) and mitotic index (continuous lines) were measured at various time points. Open circles represent the values in STA_2-free media and closed circles those in media conatining 10^{-5}M STA_2. Values are an average of 6 rats. The statistical difference was assessed by Student's t test. $^{*}p<0.02$, $^{**}p<0.01$ vs the respective control values.

FIGURE 5. Analysis of the Proliferative Process in G1/S-Synchronized VSMC

The cells were synchronized in the G1/S boundary according to the method described in the text. Hydroxyurea was washed out with media containing 10% serum at zero time. (^{3}H)Thymidine uptake (broken lines) and mitotic index (continuous lines) were measured at various time points. In the upper graph (a), open circles represent the values in STA_2-free media and closed circles those in media containing 10^{-5}M STA_2. In the lower graph (b), open circles represent the values in PDGF-free media and closed circles those in media containing 10^{-2}U/ml PDGF. Values are an average of 6 rats. Group differences were assessed by Student's t test. $^{*}p<0.05$, $^{**}p<0.01$ vs the respective control values.

FIGURE 6. Effects of STA_2 on the Mitotic Process in Colchicine-treated VSMC

The cells were synchronized in the premitotic period according

to the method described in text. Colchicine was washed out at zero time with fresh media containing 10% serum. Mitotic index was measured at various time points. The mitotic index of VSMC cultured in STA_2-free media is shown by open circles, and that in media with STA_2 is shown by closed circles. Values are an average of 5 rats. The difference was assessed by Student's t test. $^{*}p<0.05$, $^{**}p<0.01$ vs the respective controls.

FIGURE 7. Effect of STA_2 on Incorporation of (^{3}H)Leucine into the Proteins of VSMC

The left graph shows the effect of STA_2 on the incorporation of (^{3}H)leucine into VSMC during G0/G1 period. Quiescent VSMC were incubated in 10% FBS containing a given concentration STA_2. After 12 hours, the radioactivity in cellular protein was determined according to the method described in text. The right graph illustrates the incorporation of (^{3}H)leucine during G2/M period. G1/S-synchronized VSMC were cultured for 12 hours in 10% sera with a given concentration of STA_2. Values are an average of 6 rats. Values are corrected for protein contents of the cells before the experiment, which are also found related to the cell numbers.

FIGURE 8. Effect of STA_2 on Actin Formation

The left graph shows the results from SDS-PAGE analysis of the actin formation during G2/M period. The cells, synchronized in the G1/S boundary, were stimulated with 10% fresh sera. After 12 hours, the homogenate was analyzed by SDS-PAGE and the contents of actin was

measured by a densitometric assay method as described in text. Rm represents the relative mobility of each peak. OD_{517}xdRm represents the integrated absorbance. The right graph shows the incorporation of (^{3}H)leucine into the actin molecules. Radioactivity in the band corresponding to standard actin was determined. Values are an average of 6 rats. Values are corrected for protein contents of the cells before the experiment, which are also found related to the cell numbers.

FIGURE 9. Inhibitory Effects of Puromycin on Mitotic Process

VSMC, synchronized in the G1/S boundary, were stimulated at zero time with fresh 10% sera containing a given concentration of puromycin. The mitotic index was determined at various time points. Open circles represent VSMC in puromycin-free media. The mitotic index curve was shifted toward the right, as indicated by open squares (10^{-8} M puromycin) and triangles (10^{-6} M puromycin). Values are an average of 6 rats. $^{*}p<0.05$, $^{**}p<0.01$ vs the respective control values.

FIGURE 10. Retardation of the Mitotic Process by Cytochalasin D

G1/S-synchronized VSMC were stimulated at zero time with fresh 10% sera. Mitotic index was determined at various time points. Closed marks represent VSMC in media containing 10^{-5}M STA_2. Squares represent VSMC in media containing 10^{-7}M cytochalasin D. Values are an average of 6 rats. CD represents cytochalasin D. F values were 21.2 for control and CD ($p<0.001$) and 24.8 for STA_2 and STA_2+CD ($p<0.001$). $^{*}p<0.05$, $^{**}p<0.001$ vs the respective values in STA_2. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs the respective values in Control.

TABLE 1. Effects of STA_2 on (^3H)Thymidine Uptake by Randomly Proliferating VSMC

Concentrations	(^3H)Thymidine Uptake*
0 (moles/L)	10.8 ± 1.3
10^{-8}	13.5 ± 1.2
10^{-6}	14.3 ± 0.6
10^{-4}	16.4 ± 1.4
Difference**	$p<0.05$

10^4 cells were inoculated and cultured in media containing 10% sera for 3 days. The randomly cycling VSMC were stimulated in fresh 10% sera containing a given concentration of STA_2 for 24 hours. (^3H)thymidine incorporated into DNA fragments was measured according to the method described in the text. Values are an average of 6 rats. *Unit is expressed as $x10^{-3}$ dpm/10^4 cells/24 hours. **Difference was assessed by one way analysis of variance. F value was 3.12.

TABLE 2. Dose-Dependency of the Stimulation of Cell Division by STA_2

Concentrations	Mitotic Index
0 (moles/L)	24.7 ± 2.9 (6) (%)
10^{-8}	25.0 ± 2.0 (6)
10^{-7}	28.7 ± 0.9 (6)
10^{-6}	31.3 ± 2.3 (6)
10^{-5}	35.7 ± 2.6 (6)
Difference*	$p<0.05$

The mitotic index of G1/S-synchronized VSMC was measured at 8 hours after washout of hydroxyurea. Number in parentheses represents number of rats. *Difference was assessed by one way analysis of variance. F value was 3.32.

FIGURE 1.


Standard
Actin
SMC 1/1
SMC 1/3
Aortic Wall
200kD
116kD
97kD
66kD
43kD

FIGURE 2.

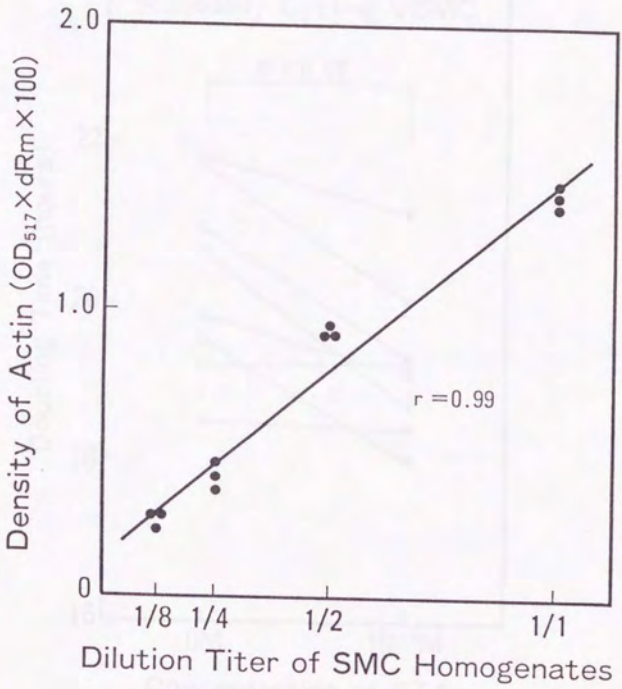

2.0
1.0
0
Density of Actin ($OD_{517} \times dRm \times 100$)
r =0.99
1/8
1/4
1/2
1/1
Dilution Titer of SMC Homogenates

FIGURE 3.

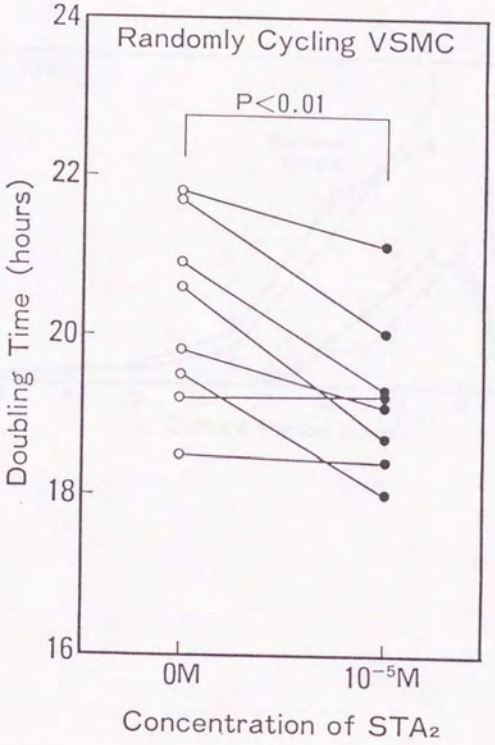

Randomly Cycling VSMC
P<0.01
24
22
20
18
16
Doubling Time (hours)
0M
10⁻⁵M
Concentration of STA₂

FIGURE 4.

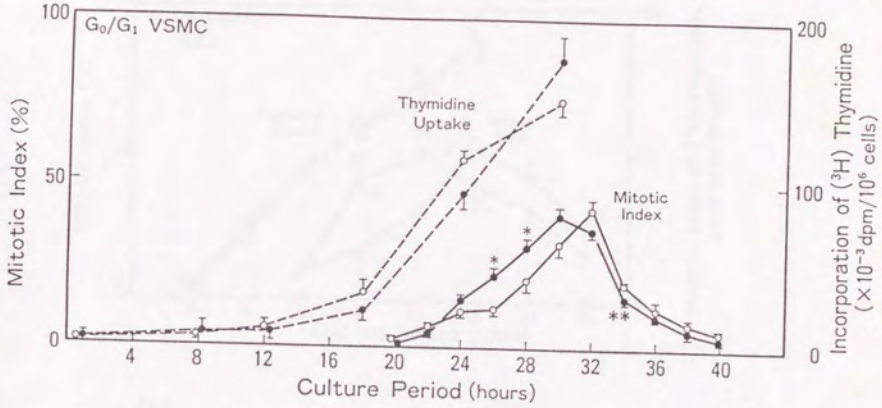

G_0/G_1 VSMC
Thymidine Uptake
Mitotic Index
Mitotic Index (%)
Incorporation of (^{3}H) Thymidine ($\times 10^{-3}$dpm/10^6 cells)
Culture Period (hours)
100
50
0
200
100
0
4
8
12
16
20
24
28
32
36
40
*
*
**

FIGURE 5.

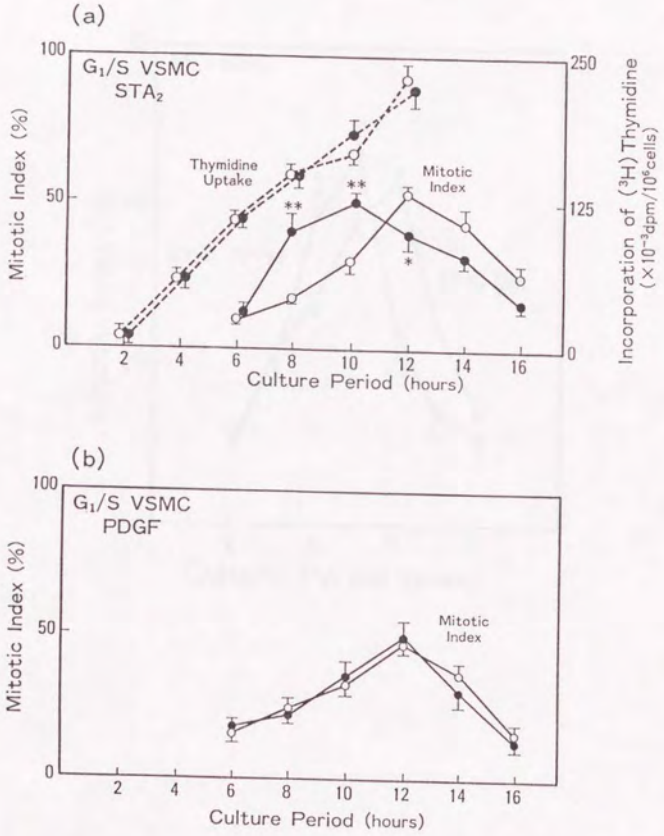

(a)
G1/S VSMC
STA2
Thymidine Uptake
Mitotic Index
Mitotic Index (%)
Incorporation of (3H) Thymidine (×10⁻³dpm/10⁶cells)
Culture Period (hours)
(b)
G1/S VSMC
PDGF
Mitotic Index
Mitotic Index (%)
Culture Period (hours)

FIGURE 6.

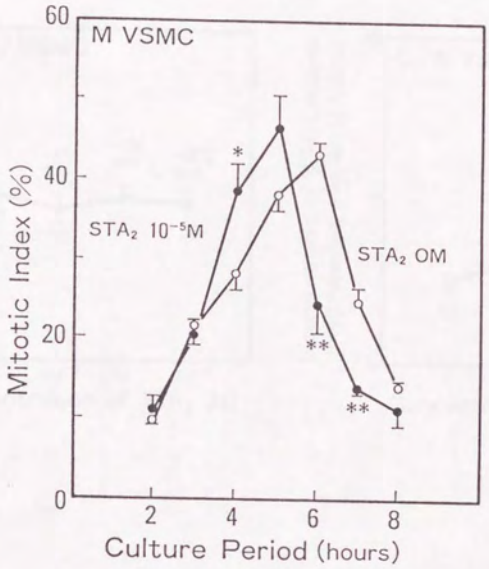

M VSMC
Mitotic Index (%)
60
40
20
0
2
4
6
8
Culture Period (hours)
STA₂ 10⁻⁵M
STA₂ OM
*
**
**

FIGURE 7.

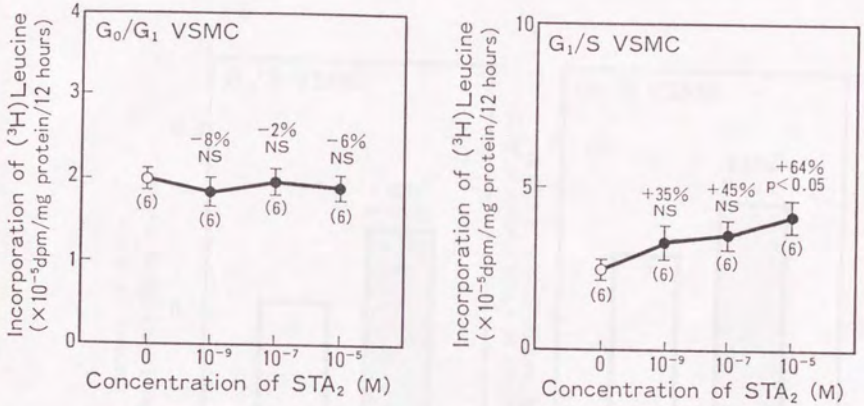

G0/G1 VSMC
Incorporation of (3H)Leucine (×10-5dpm/mg protein/12 hours)
−8% NS
−2% NS
−6% NS
(6)
(6)
(6)
(6)
(6)
0
10-9
10-7
10-5
Concentration of STA2 (M)
G1/S VSMC
+35% NS
+45% NS
+64% P<0.05
(6)
(6)
(6)
(6)
(6)

FIGURE 8.

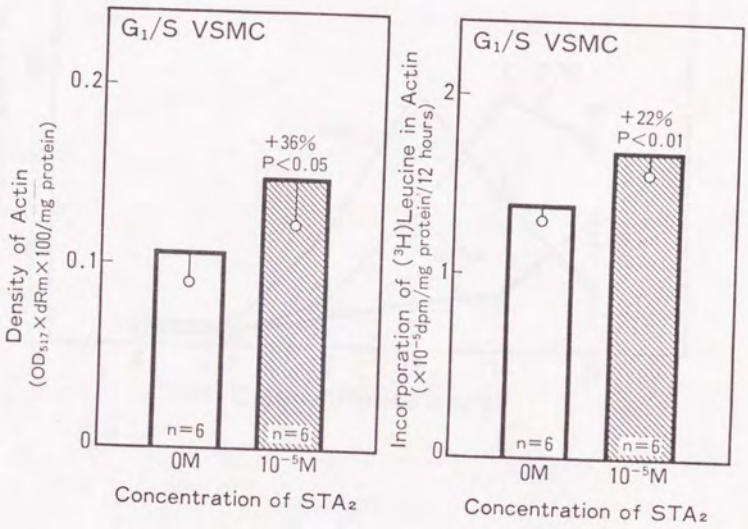

G1/S VSMC
Density of Actin
(OD517×dRm×100/mg protein)
0.2
0.1
0
+36%
P<0.05
n=6
n=6
0M
10−5M
Concentration of STA2
G1/S VSMC
Incorporation of (3H)Leucine in Actin
(×10−5dpm/mg protein/12 hours)
2
1
0
+22%
P<0.01
n=6
n=6
0M
10−5M
Concentration of STA2

FIGURE 9.

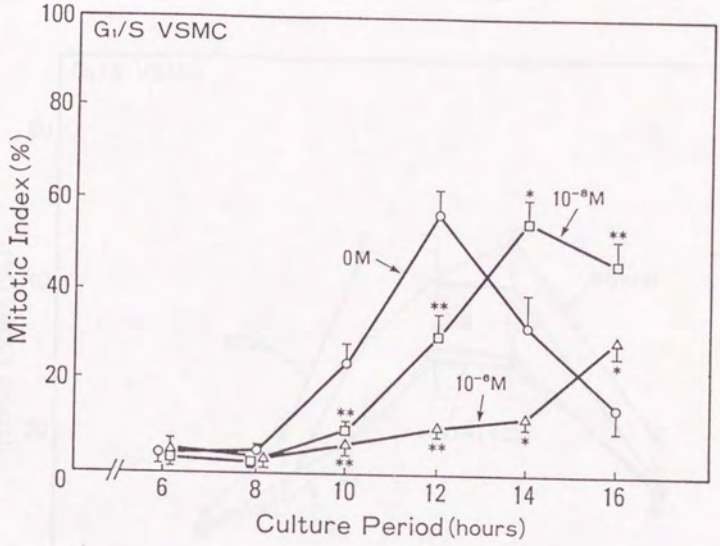
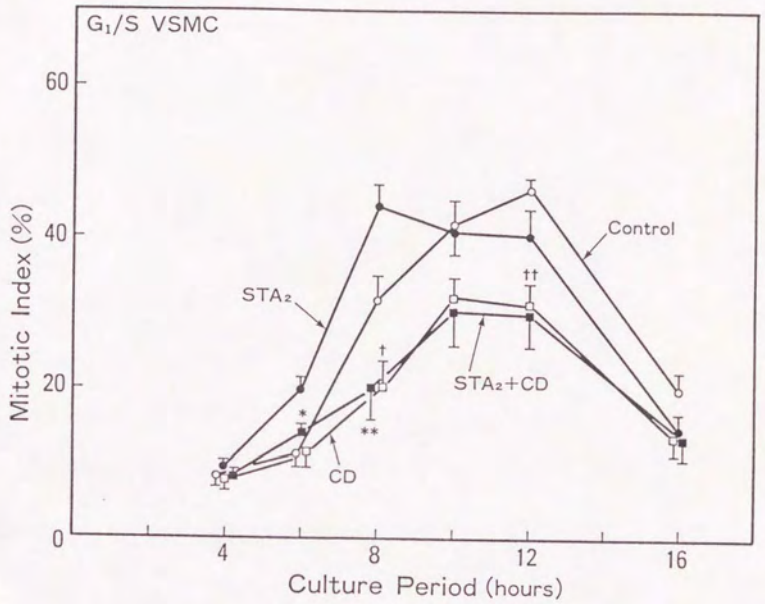
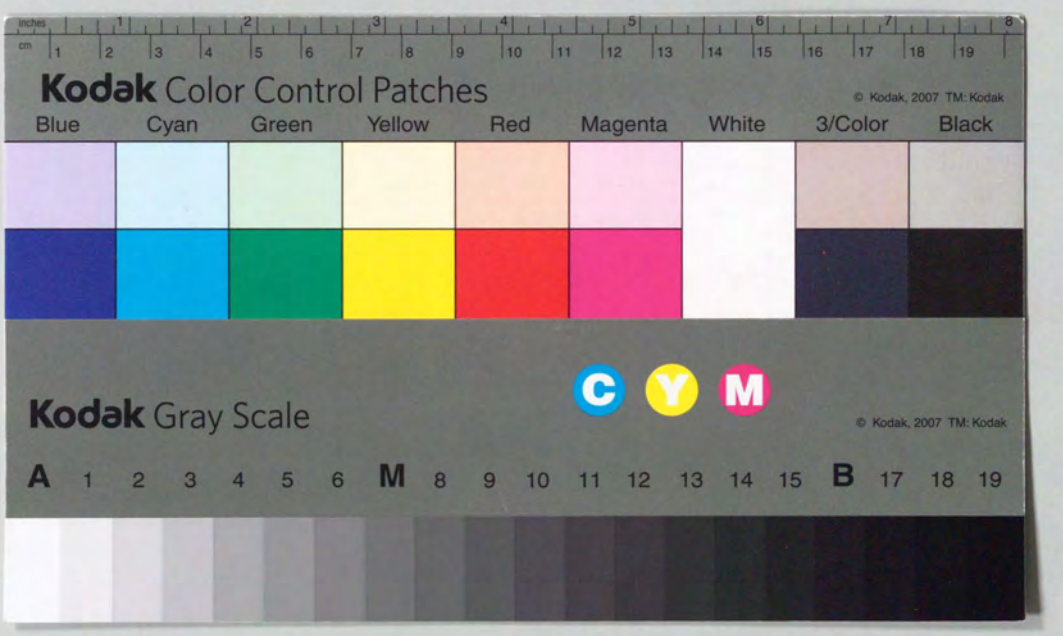

G1/S VSMC
Mitotic Index (%)
100
80
60
40
20
0
6
8
10
12
14
16
Culture Period (hours)
0M
10⁻⁸M
10⁻⁶M
*
**

FIGURE 10.


G1/S VSMC
60
40
20
0
Mitotic Index (%)
4
8
12
16
Culture Period (hours)
Control
STA2
STA2+CD
CD
*
**
†
††


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19