Effect of Various Plasminogen Activators on Prostacyclin Synthesis in Cultured Vascular Cells

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In this study, we examined the effects of various plasminogen activators on arachidonic acid release and prostacyclin biosynthesis in cultured rat aortic smooth muscle cells and bovine pulmonary artery endothelial cells. Prostacyclin was the major product formed from arachidonic acid in aortic smooth muscle cells and endothelial cells. When intact cells were incubated with streptokinase, one of the plasminogen activators, a significant stimulatory effect on prostacyclin biosynthetic activity in cells was evident without any cellular damage at all concentrations used (1-5,000 units/ml). Streptokinase also caused a marked release of arachidonic acid. However, when it was incubated with cell-free homogenates and [3H]arachidonic acid, it did not show any effects on prostacyclin biosynthesis. The addition of urokinase and tissue-type plasminogen activator had no effect on prostacyclin biosynthesis. Urokinase stimulated the release of arachidonic acid from cells, but it did not show any effect on prostacyclin release at any concentration of urokinase (1-5,000 units/ml). The release of arachidonic acid and the increased prostacyclin synthesis were not observed when tissue-type plasminogen activator was added. These results indicate that, among various plasminogen activators investigated, only streptokinase causes the release of arachidonic acid and prostacyclin. This could be a beneficial effect in thrombolytic therapy. (Circulation Research 1988; 63:1029-1035)

The cascade of arachidonic acid to prostacyclin (PGI2) in blood vessels and to thromboxane A2 in platelets has been implicated in control of thrombus formation, which plays a very important role in atherosclerosis and myocardial infarction.1,2 This is strongly supported by the data that prostacyclin generation decreases in the mesenteric artery and aorta while thromboxane A2 generation increases in platelets during the development of experimental atherosclerosis in rabbits.3 Taking this into consideration, PGI2 and its stable analogues would appear to be potentially valuable therapeutic agents. By virtue of the vasodilatory properties of these agents and their ability to inhibit platelet activation by virtually all aggregating stimuli,4 PGI2 analogues may be effective antithrombotic agents in vivo. It was demonstrated that PGI2 infusion in the anesthetized dog was effective in the prevention of coronary artery thrombosis.4 Furthermore, administration of a low dose of PGI2 concomitantly with a phosphodiesterase inhibitor potentiated the antithrombotic effects of PGI2 without the decrease in blood pressure associated with higher doses of the prostanoïd.5 During the past several years, a number of studies have demonstrated that acute transmural myocardial infarction is frequently associated with coronary thrombosis6 and that the prompt administration of a thrombolytic agent, such as streptokinase, may reestablish anterograde flow in the involved coronary artery.7,8 Some studies have suggested that timely reperfusion may improve left ventricular function9 and survival10 over the several months after the acute event.

The beneficial effect of plasminogen activator infusion can be due to a variety of mechanisms. One way to reduce infarct size is to increase collateral coronary flow by the use of vasodilators during the ischemic period. The effects of plasminogen activators on coronary vascular tone were therefore tested in isolated perfused cat coronary arteries in vitro. It was found that neither the vehicle nor tissue-type plasminogen activator exhibited any vasodilator properties in very high concentrations.11 This is in contrast to streptokinase, which was shown to exert vasodilator actions on isolated arteries and veins.12,13
However, no data have been available on the effects of plasminogen activators on prostacyclin biosynthesis in the vascular wall. In this study, we attempt to determine the effect of various plasminogen activators on PGi, synthesis in cultured aortic smooth muscle cells and endothelial cells.

Materials and Methods

Cell Culture

Aortic smooth muscle cells of Wistar rats 3 to 4 weeks old were isolated from medial explants essentially as described earlier. Briefly, aortas were cut open to expose the intimal surface. Intima and adventitia were stripped off with gauze and a razor blade, and the explants were then cut into sections approximately 4 mm thick. The intimal sides of explants were attached to Petri dishes. When the explants adhered to the dishes, Dulbecco’s Modified Eagle's Medium (DMEM; Flow Laboratories, Rockville, Maryland) supplemented with 20% fetal bovine serum (FBS) from Flow Laboratories was carefully added. The dishes were then incubated at 37°C in a humidified 5% CO2-95% air atmosphere. Cells grown to confluence were subcultured at 1.2X10^6 cells/dish in 2 ml DMEM containing 0.3% FBS in 35-mm dishes. Cultures established in this manner were used throughout the course of the study. Cells from passages 10–35 were used in this series of experiments. The quality of this cell line was checked by measurement of the prostacyclin synthesis. Usually, the synthesis of prostacyclin in cells from passages 10–35 was stable.

Aortic endothelial cells (bovine pulmonary artery) were obtained from Flow Laboratories and maintained in Minimum Eagle’s Medium supplemented with 20% FBS. The subculture of pulmonary artery endothelial cells was the same as described above. The quality of this cell line was also checked as aortic smooth muscle cell by measurement of the prostacyclin synthesis.

Prostaglandin Synthesis

Triplicate cultures of 1.2X10^6 cells plated in 2 ml DMEM containing 0.3% FBS and 1 μCi of [3H]arachidonic acid (135 Ci/mol; Radiochemical Centre, Amersham, England) in 35-mm Falcon plastic dishes were cultured for 24 hours. The incorporation of [3H]arachidonic acid into aortic smooth muscle cells and pulmonary artery endothelial cells was 22±6% and 22±2%, respectively. The cells on the dish were washed three times with 2 ml of the medium and then incubated at 37°C with the indicated concentrations of each plasminogen activator (streptokinase [SK], Sigma Chemical, St. Louis, Missouri; urokinase [UK], Fujisawa, Japan; tissue-type plasminogen activating factor [tPA], Kowa, Japan) in 0.8 ml of serum-free medium. The lipids released into the medium from cells were extracted with 2 ml ethyl acetate and analyzed.

To separate prostaglandin (PG) F20 and 6-keto-PGF1α, the plates were first allowed to develop up to 15 cm from the origin and, after drying, were again allowed to develop up to 17 cm from the origin in a solvent system containing the organic phase from ethyl acetate/acetic acid/2,4,trimethyl pentane/water (110:20:50:100 vol/vol). The zones corresponding to authentic prostaglandins (PGF2α, PGF2β, and 6-keto-PGF1α, Sigma) and arachidonic acid were scraped and counted in a liquid scintillation counter. Further identification of the reaction products was done using a method involving high performance liquid chromatography. The retention times of 6-keto-PGF1α, thromboxane B2, PGF2α, and PGE2 were 3, 5, 7, and 12 minutes, respectively.

The release of PGi, from cultured aortic smooth muscle cells was also determined by radioimmunoassay of 6-keto-PGF1α. Aortic smooth muscle cells (2×10^5 cells/dish) were cultured as described for the assay of phospholipid hydrolysis, except that no tritiated arachidonic acid was used. Cells were incubated with each plasminogen activator at 37°C for 2 hours. The medium was collected and extracted with ethyl acetate as described above. Then, 6-keto-PGF1α was measured by radioimmunoassay as described earlier.

Prostacyclin Synthesis by Homogenates of Aortic Smooth Muscle Cells and Endothelial Cells

The confluent cells were washed three times with 0.1 M Tris-HCl, pH 8.0, and then scraped from the dishes. Cells were sonicated, and the resulting homogenates were used for the prostaglandin synthesis assay. The homogenate (500 μg protein) was incubated with 0.2 μCi [3H]arachidonic acid for 15 minutes at 37°C in 200 μl Tris-HCl, pH 8.0. Prostaglandins were extracted with ethyl acetate at pH 3.5 and separated by thin-layer chromatography (TLC).

Phospholipase Assay

Cells prelabeled with [3H]arachidonic acid were washed on the dishes three times with fresh medium and then incubated with the indicated concentrations of plasminogen activators in DMEM for up to 60 minutes at 37°C. The lipids in the medium were extracted with ethyl acetate. The solvent was evaporated in a vacuum, and the residues were applied to TLC and developed.

When cellular lipids were obtained for TLC, the medium was removed, the cells were rapidly washed twice with 2 ml of medium, treated with 1 ml of 70% methanol, and then scraped from the dishes. The lipids were extracted by the method of Folch et al. Neutral lipids were separated by TLC with the solvent system described below. TLC was developed in isopropyl ether/acetic acid (96:4 vol/vol), and then again in a solvent system containing petroleum ether/diethyl ether/acetic acid (90:10:1 vol/vol). The respective spots (monoacylglycerol [MG], diacylglycerol [DG], triacylglycerol [TG], arachi-
donic acid; Sigma) were scraped off and counted in a scintillation spectrometer.

Cytotoxicity Assay

Cells were cultured in the same manner as described for the assay of prostaglandin synthesis, except that no tritiated arachidonic acid was used. Cell viability was assessed by the ability to exclude 0.023% trypan blue dye for 2 minutes at room temperature with an inverted microscope with phase optics. After treatment of aortic smooth muscle and endothelial cells with each plasminogen activator for 60 minutes, the medium (600 μl) was removed and centrifuged to remove any nonadherent cells, and aliquots (250 μl) were tested for β-galactosidase and lactate dehydrogenase activities.

Statistical Analysis

Student's t test was used to analyze the results. Significance was considered as p<0.05. Data are presented as mean±SEM.

Results

Effect of Plasminogen Activators on Prostacyclin Synthesis

Cultured pulmonary artery endothelial cells and aortic smooth muscle cells previously biosynthetically labeled by incorporation of [3H]arachidonic acid were incubated with SK, UK, or tPA in serum-free medium for 90 minutes. The results are shown in Figure 1. SK stimulated the release of [3H]6-keto-PGF1α at a concentration of 100 units/ml in both types of cells. The maximal stimulation by SK was observed at 5,000 units/ml. The amount of released 6-keto-PGF1α was higher in pulmonary artery endothelial cells than in smooth muscle cells at all doses used (p<0.001). In contrast, the stimulation of 6-keto-PGF1α was not observed by the additions of either UK or tPA in any of cell types used. As shown in Figure 2, SK caused the gradual release of 6-keto-PGF1α. The increase in this labeled 6-keto-PGF1α was detectable within 10 minutes and reached a plateau at 60 minutes. UK (1,000 IU/ml) and tPA (1,000 IU/ml) did not cause detectable 6-keto-PGF1α release within the 90-minute incubation.

The amount of prostacyclin released into the medium was also measured by radioimmunoassay for 6-keto-PGF1α (Figure 3). The dose-response curve of 6-keto-PGF1α release was almost the same as the tritium-labeled pattern. SK at concentrations ranging from 100 units/ml to 5,000 units/ml stimulated prostacyclin production significantly (p<0.001 compared with control).

Effect of Plasminogen Activators on Arachidonic Acid Release

To study the mechanism of stimulation of 6-keto-PGF release by SK, the effect of plasminogen activator on arachidonic acid was determined because arachidonic acid is a substrate for PG synthesis, and it is known that phospholipase is a rate-limiting step for synthesis of PGs. SK stimulated the release of [3H]-arachidonic acid in a dose-dependent manner. The dose curve for the release of [3H]-arachidonic acid was similar to that for the release of prostacyclin. The release of arachidonic acid in PAEC was higher than in smooth muscle cells at all doses (p<0.001) (Figure 4).

The time course for the release of arachidonic acid was similar to that of prostacyclin release by SK stimulation (Figure 5). These results indicate that SK in the range from 100 units/ml to 5,000 units/ml stimulates prostacyclin production is due to the activation of phospholipase. UK also stimulated arachidonic acid release, but no PGs were detected in our system. These results suggest that UK cannot activate PGs synthesis in these systems.
However, tPA did not stimulate the arachidonic acid release at any concentration used.

Effect of Plasminogen Activators on Prostacyclin Synthesis by Cell-Free Homogenates

The extent of prostacyclin synthesis in the cell-free homogenates was investigated by exogenously adding [3H]arachidonic acid. The major product was prostacyclin, and a tracer amount of PGE2 was formed from [3H]arachidonic acid in homogenates. The conversion rates of [3H]arachidonic acid to prostacyclin in pulmonary artery endothelial cells and smooth muscle cell homogenates were 12.6±3.1 and 8.1±1.0%, respectively. In culture cells, PG synthesis was not observed in the addition of UK or tPA. To clarify the effect of UK and tPA on PG synthetase, UK or tPA was incubated with cell homogenates. As shown in Table 1, addition of plasminogen activators directly to the homogenates did not significantly change the conversion of arachidonic acid to prostacyclin. UK and tPA did not inhibit PG synthesis, nor did tPA in the cell-free system. The effects of PG release by UK in cells were not clarified in these experiments.

Cytotoxicity

The effects of plasminogen activators on the release of cellular lactate dehydrogenase and β-galactosidase activity into the external medium were determined. Lactate dehydrogenase and β-galactosidase activities are highly compartmentalized in the cytoplasm and lysosomes, respectively, and have proved to be useful markers for damage to cell surface and lysosomal membranes. The release of cellular lactate dehydrogenase and β-galactosidase activities into the external medium by the treatment of SK (1–5,000 IU/ml), UK (1–5,000 IU/ml), and tPA (1–5,000 IU/ml) were not observed. Cell viability was also determined by the ability of trypan blue exclusion from cells. Cells were not stained at any concentrations of the plasminogen activators.
activators used. These results show that the release of arachidonic acid and 6-keto-PGF\textsubscript{1α} are not due to phospholipase activation that resulted from cell damage caused by SK and UK.

Discussion

Thrombolytic therapy has become a widely used method for the treatment of acute myocardial infarction. Streptokinase is one of the enzymes commonly used to activate the thrombolytic system in order to dissolve the clot. At present, it is not certain whether survival of patients receiving thrombolytic therapy is longer than survival of those patients receiving more conventional modes of treatment. However, successful thrombolysis has been associated with significant increases in the left ventricular ejection fraction. In these studies, the greatest changes in ejection fractions were seen in short-term comparisons of ejection fractions before and immediately after streptokinase therapy. Studies done over a longer time have shown a much smaller improvement in the ejection fraction. These findings suggest the beneficial effect of recanalization of the occluded thrombus by fibrinolytic agents. However, the effect of SK itself on left ventricular function in the ischemic myocardium after reperfusion is not clear. Fung and Rabkin\textsuperscript{28} suggested that SK had either a protective effect on myocardial cell function during the ischemic process or a property that inhibits myocardial damage in the early phase of reperfusion injury by using an isolated heart preparation. In contrast, Jansens and Verhaegh\textsuperscript{29} found that streptokinase depressed the response to adrenergic nerve stimulation in isolated canine systemic arteries and veins. Furthermore, this inhibition of adrenergic neural effects was not found to be due to the stimulation of prejunctional cholinergic, dopaminergic, or histaminergic receptors or to generation of local mediators. Thus, while the potential for SK to exert effects independent of those on the fibrinolytic system has been clearly demonstrated, the nature of these effects and the underlying mechanism responsible for them are not yet clear.

On the other hand, prostacyclin is a vasodilator and inhibitor of platelet aggregation that is synthesized by a variety of tissues, including isolated perfused hearts, blood vessels, and vascular endothelial and smooth muscle cells grown in tissue culture.\textsuperscript{29-32} Thromboxane A\textsubscript{2}, an arachidonic acid metabolite produced by platelets, promotes aggregation and is a potent vasoconstrictor.\textsuperscript{33} Recently, it has been suggested that the balance between PGI\textsubscript{2} produced by blood vessels and thromboxane A\textsubscript{2} produced by platelets, both of which are transformed from the same substrate, arachidonic acid, plays an important role in thrombus formation and cardiovascular disease.\textsuperscript{33} A potentially valuable use for PGI\textsubscript{2} and its analogues would be as an adjunct to thrombolytic therapy. It was recently reported that during coronary artery thrombolysis with SK in the anesthetized dog, infusion of PGI\textsubscript{2} produced an augmentation in the thrombolytic effectiveness of streptokinase.\textsuperscript{34} These findings are significant in light of the fact that reocclusion of the coronary artery is a frequent occurrence after recanalization of the obstructed vessel by thrombolysis.

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<th>TABLE 1. Prostacyclin Synthesis in SMC and PAEC Homogenates</th>
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<td>Prostacyclin synthesis (%)</td>
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<td>SMC (n=9)</td>
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*The conversion rates from arachidonic acid to 6-keto-PGF\textsubscript{1α}. SMC, aortic smooth muscle cells; PAEC, pulmonary artery endothelial cells; n, number of experiments; and NS, not significant.
In the present study, the effect of plasminogen activators on prostacyclin synthesis in aortic smooth muscle cells and aortic endothelial cells was determined to investigate whether these agents have effects on these cells other than fibrinolytic effects and to find one of the possible mechanisms of the vasodilator effect induced by SK. Since 6-keto-PGF$_{1\alpha}$ is the major stable product of prostacyclin, and since prostaglandins are not stored in cells, the data indicate that SK stimulates the synthesis of prostacyclin by both cultured smooth muscle cells and pulmonary artery endothelial cells. Incubation of the cells with SK produced a significant dose-dependent accumulation of 6-keto-PGF$_{1\alpha}$ in culture media. SK also induced a dose-dependent increase in the release of arachidonic acid from these cells into the culture medium. The amount of arachidonic acid release and 6-keto-PGF$_{1\alpha}$ release induced by SK were equivalent. However, the magnitude of the aortic smooth muscle cell arachidonic acid release induced by SK was significantly higher than that of 6-keto-PGF$_{1\alpha}$ produced by SK at the same concentration. These results indicate that SK stimulates the release of 6-keto-PGF$_{1\alpha}$ from these cells in tissue culture, and that increased 6-keto-PGF$_{1\alpha}$ synthesis is likely to be a consequence of the increased availability of free arachidonic acid. This conclusion is supported by the fact that the addition of SK directly to the homogenates did not have any effect on prostacyclin biosynthesis. This result indicates that the stimulatory effect of SK on prostacyclin release in intact cells is not caused by the activation of cyclooxygenase. In contrast, UK stimulated arachidonic acid release but not prostacyclin release. UK had no effect on prostacyclin synthesis in the cell-free incubation system. This is an unusual finding, because released arachidonic acid was not used for prostacyclin synthesis. These results suggest that UK may interfere with the transport of released arachidonic acid by fatty acid binding protein, which has an important role in fatty acid movement through the cytosol from plasma membrane to endoplasmic reticulum, or that it may stimulate some feedback system. Another possibility is free radicals that are generated during membrane destruction. During this process, the cyclooxygenase is irreversibly deactivated. In our experiments, it is not clear whether UK stimulates these inhibitory processes of prostacyclin synthesis. Further investigations in this area are required to clarify these mechanisms.

The present study indicates that, in addition to its fibrinolytic properties, SK has an effect on prostacyclin release in aortic smooth muscle cells and pulmonary artery endothelial cells. Although extrapolation of experimental data to man must be done with caution, the results of this study may have therapeutic implications. Further studies are required under more physiological conditions in vivo.

Acknowledgment

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**KEY WORDS**  
phospholipase • urokinase • streptokinase • tissue-type plasminogen activator • prostacyclin synthesis
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