Coagulant Factor Xa Inhibits Prostacyclin Formation in Human Endothelial Cells

Role of Factor V


Thrombin stimulates prostacyclin formation in cultured human endothelial cells. However, a countervailing process that prevents prostacyclin overproduction has not been described previously. In this study, we demonstrate that Factor Xa can inhibit prostacyclin synthesis induced by thrombin or sodium arachidonate. The required concentration of Factor Xa represents activation of only 2% of the Factor X in plasma. The inhibition is reversed by a human monoclonal antibody directed against the light chain of Factor Va (M, = 78,000), which suggests that Factor Va is required for this down-regulation of prostacyclin production. Confluent human endothelial cells (10^6) contained 1.4 to 2.2 μg of Factor V antigen as measured by a competitive enzyme-linked immunosorbent assay. The results indicate that in endothelial cells Factor Xa may play a regulatory role in prostacyclin formation through interaction with Factor Va. (Arteriosclerosis 5:244–249, May/June 1985)

Prostacyclin (PGI₂), the major prostanoid produced by endothelial cells, is the most potent inhibitor of platelet aggregation currently known. In human endothelial cells, the autacoid is produced when these cells are stimulated by a physiological agonist like thrombin or by model compounds such as ionophore A23187, both of which liberate arachidonic acid from membrane phospholipids. The liberated fatty acid is subsequently converted to prostaglandin G₂ (PGG₂) and then to prostaglandin H₂ (PGH₂) by cyclooxygenase. PGH₂ is subsequently converted to PGI₂ by prostacyclin synthetase. When sodium arachidonate or PGH₂ is added to endothelial cells, it is converted to PGI₂ through the above pathway.5

Despite its potential importance as an antithrombotic prostanoid, PGI₂ produces undesirable side effects, such as interference with normal hemostasis due to inhibition of platelet aggregation, and hypotensive disorders. Although the details of the biosynthetic pathway leading to the formation of PGI₂ are known, the regulatory mechanisms controlling the production of PGI₂ in vivo are not well understood.

We report here that the plasma proteolytic enzyme, Factor Xa is a potent inhibitor of PGI₂ synthesis in human endothelial cells grown in tissue culture. Factor Xa is generated from its zymogen, Factor X, during activation of both the intrinsic and extrinsic pathways of blood coagulation. This inhibition requires Factor V or Va, a coagulation protein synthesized in endothelial cells.

Methods

Cell Culture

Primary cultures of human endothelial cells from umbilical veins were prepared and grown to confluence (usually 4 days) at 37°C in 5% CO₂ atmosphere with Medium 199 containing 10% fetal calf serum in 24-mm plastic wells (Falconware) as described by Gimbrone. At confluence, the endothelial monolayers contained approximately 5 × 10⁵ cells per well.

From the Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania.

This work was presented at the Annual Meeting of the Federation of American Societies for Experimental Biology, San Francisco, California and published in abstract form. (Fed Proc 1983;42:2093)

This work was supported in part by Grant HL-27189 and by SCOR Thrombosis Grant HL-14217 from the National Heart, Lung, and Blood Institute.

Address for reprints: Asru K. Sinha, Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania 19140.

Received July 20, 1984; revision accepted January 3, 1985.
Incubation Procedures

Immediately before treating the cell monolayers with various substances, we poured off the spent medium from the culture wells and washed the cells four times with 2 ml of the fresh (but serum-free) medium buffered with 15 mM Hepes buffer (pH 7.2). After the final washing, 0.5 ml of the same medium was added to each well and different concentrations of thrombin or sodium arachidonate were added and incubated for 5 minutes at 37°C. After incubation, 0.5 ml of ice-cold HCl was added to the culture (final concentration 1.0 N). The PGI$_2$ content of the medium was then determined.

To determine the effect of Factor Xa on the formation of PGI$_2$ stimulated by thrombin or sodium arachidonate, various concentrations of the procoagulant were added to culture fluid overlying the monolayer and were incubated for 5 minutes at 37°C before the addition of stimulatory agents. After the stimulatory agents were added to the culture medium, the cells were incubated for another 5 minutes at 37°C and the formation of PGI$_2$ was determined. The production rate of PGI$_2$ was steady for at least the first 5 minutes of incubation. Unless otherwise indicated, PGI$_2$ formation is given as per minute of incubation.

In some experiments, attempts were made to reverse the effect of Factor Xa by washing the incubated cells with buffered culture medium. The medium containing Factor Xa was poured out of the culture well and was replaced with culture medium without the enzyme. After incubation for 5 minutes at 37°C, the medium was decanted; this procedure was repeated three times. After the final washing, the cells were treated with 24 nM thrombin as described above. PGI$_2$ formation was determined after a 5-minute incubation at 37°C.

When the cells were treated with anti-Factor V antibody, 50 μg of the antibody/ml of the culture was added to the culture medium, and the cells were incubated for 30 minutes at 37°C before addition of Factor Xa or thrombin. When both Factor Xa and thrombin were added to the culture medium of cells previously treated with the antibody, Factor Xa was added to the incubation medium before the addition of thrombin. Parallel experiments were also done with the antibody alone. The formation of PGI$_2$ was assayed after the medium was treated with HCl.

Assay of PGI$_2$

PGI$_2$ is an unstable compound which readily converts into 6-keto-prostaglandin F$_{1 \alpha}$ with dilute HCl. In the present study, PGI$_2$ was determined in its 6-keto-PGF$_{1 \alpha}$ form by radioimmunoassay with a kit developed by New England Nuclear, Boston, Massachusetts. Before assay, eicosanoids from the HCl-treated media were extracted by the method of Kent et al. Quantities of Factor V antigen as small as 10 ng could be measured by this procedure. The Factor V antigen content of the spent medium was similarly assayed. Because of the possibility that the spent medium might contain too little of the antigen to be within the assay limit of the ELISA procedure, 10 ml of the above medium was concentrated to 1 ml by ultrafiltration (AMICON-PM 30) before the assay.

Anti-Factor V Antibody

The anti-Factor V antibody used in these studies was a purified IgG fraction of a human monoclonal IgG$_{A}$, anti-Factor V antibody kindly supplied by Helen Glueck of the University of Cincinnati, Cincinnati, Ohio. Typically, the globulin fraction of the antisera was precipitated by 33% (NH$_4$)$_2$SO$_4$ saturation. The precipitated fraction was dissolved in water and further purified by DEAE-cellulose ion exchange chromatography with use of a 0.01 M phosphate buffer (pH 8.0). The antibody fraction was then purified by protein A-Sepharose affinity chromatography. When the resulting IgG (40 μg) was incubated with 1 ml of normal plasma containing one coagulant unit of Factor V for 30 minutes at 20°C, more than 99% of the Factor V affinity was neutralized. The specificity of this antibody was established by Wilson et al. This antibody binds both Factor V and Factor Va and recognizes an antigenic determinant on the Factor Va light chain (M, = 78,000). Control experiments were run by using normal human IgG (100 μg/ml) under identical conditions.

Materials

Human thrombin was purified by the method of Fenton et al. with commercial thrombin used as the starting material (Sigma Chemical Company, St. Louis, Missouri). The details of the preparation of
Factor Xa have been reported before.\textsuperscript{22} Diisopropyl fluorophosphate (\textit{iPr\textsubscript{2}}-F) inactivated Factor Xa (\textit{iPr\textsubscript{2}}-Xa) was prepared by this method,\textsuperscript{22} and \textit{6}\textsuperscript{[5, 8, 9, 11, 12, 14, 15]-H(N)}\textsubscript{3}PGF\textsubscript{1\alpha} (specific activity, 120 Ci/mmol) was obtained from New England Nuclear, Boston, Massachusetts. All other chemicals were analytical grade.

**Results**

**Stimulation of PGI\textsubscript{2} Synthesis by Thrombin and Sodium Arachidonate**

Treatment of cultured human umbilical vein endothelial cells with increasing amounts of thrombin produced increasing quantities of PGI\textsubscript{2} measured in its 6-keto-PGF\textsubscript{1\alpha} form by radioimmunoassay (Figure 1). In the presence of 12, 24, and 36 nM thrombin (1 unit = 12 nM), the basal level of PGI\textsubscript{2} increased 6-, 12-, and 16-fold, respectively, in 1 minute in these cells. The endothelial cells were maximally stimulated by 72 nM thrombin to produce approximately 18 pmol of PGI\textsubscript{2}/10\textsuperscript{7} cells during the same period of incubation.

Incubation of endothelial cells in tissue culture with sodium arachidonate also stimulated the formation of PGI\textsubscript{2} in these cells. In the presence of 2.4 and 4.8 \textmu{M} sodium arachidonate, the basal level of PGI\textsubscript{2} increased from 1.2 ± 0.25 pmol/10\textsuperscript{7} cells to 2.6 ± 0.10 and 4.4 ± 0.14 pmol/10\textsuperscript{7}, respectively, in 5 minutes at 37°C.

**Inhibition of PGI\textsubscript{2} Synthesis by Factor Xa**

Incubation of endothelial cells with increasing concentrations of blood coagulation Factor Xa for 2 minutes at 37°C before addition of thrombin progressively inhibited the production of PGI\textsubscript{2} in these cells (Figure 2). In the presence of 24 nM of thrombin alone, approximately 13 pmol of PGI\textsubscript{2}/10\textsuperscript{7} cells was produced. When these cells were treated with 1 and 2 nM of Factor Xa (1 unit of Factor X, the amount in 1 ml of normal plasma = 100 nM), the production of PGI\textsubscript{2} was inhibited by 25% and 60%, respectively. A Factor Xa concentration of 3 nM inhibited PGI\textsubscript{2} production by nearly 80%. Thus, on a molar basis, the inhibitory effect of Factor Xa was at least 10-fold more potent than the stimulatory effect of thrombin on PGI\textsubscript{2} synthesis in the endothelial cells. The inhibitory effect of Factor Xa was partially reversible. The synthesis of PGI\textsubscript{2} (12 pmol/10\textsuperscript{7} cells/minute) in endothelial cells induced by 24 nM thrombin decreased to 4 pmol/10\textsuperscript{7} cells/minute in the presence of 2 nM of Factor Xa. Washing the cells with the buffered culture medium (as described in the Methods section) increased the formation of PGI\textsubscript{2} to 9 pmol/10\textsuperscript{7} cells/minute when the cells were stimulated by the same concentration of thrombin. The inhibitory effect of Factor Xa is related to its enzymic activity; \textit{iPr\textsubscript{2}}-Xa (10 nM) did not possess any coagulant activity and had no effect on the inhibition of PGI\textsubscript{2} synthesis in endothelial cells induced by thrombin.

Factor Xa inhibited PGI\textsubscript{2} synthesis in endothelial cells in the case of thrombin, and the procoagulant also inhibited sodium arachidonate-stimulated PGI\textsubscript{2}.

---

**Figure 1.** Stimulation of PGI\textsubscript{2} formation in endothelial cells. Confluent human umbilical vein endothelial cells were incubated with increasing concentrations of thrombin for 5 minutes at 37°C. After incubation, ice-cold HCl was added to the medium (to a final concentration of 1.0 N) to stop the reaction. The medium was extracted, and the 6-keto-PGF\textsubscript{1\alpha} content of the extract was determined by radioimmunoassay. Each point represents the mean ± SEM of three experiments. The amount of PGI\textsubscript{2} formed per minute of incubation is shown.

**Figure 2.** Inhibition of PGI\textsubscript{2} synthesis in endothelial cells by Factor Xa. Human endothelial cells were incubated with different concentrations of Factor Xa for 5 minutes at 37°C before the adding of thrombin (24 nM). The experimental procedures were described in the legend to Figure 1. Each point is the means ± SEM of three experiments. PGI\textsubscript{2} formation is expressed in per minute of incubation.
INHIBITION OF PROSTACYCLIN FORMATION

Table 1. Inhibition of PGI₂ Synthesis from Arachidonic Acid in Endothelial Cells by Factor Xa

<table>
<thead>
<tr>
<th>Addition to cell culture</th>
<th>6-keto-PGF₁₀₂ (pmol/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.2 ± 0.25</td>
</tr>
<tr>
<td>Sodium arachidonate (4.8 μM)</td>
<td>4.4 ± 0.14</td>
</tr>
<tr>
<td>Sodium arachidonate (4.8 μM) + Factor Xa (10 nM)</td>
<td>2.7 ± 0.22</td>
</tr>
<tr>
<td>Factor Xa (10 nM)</td>
<td>1.1 ± 0.15</td>
</tr>
</tbody>
</table>

Values are means ± SEM of three experiments.

synthesis (Table 1). Addition of 4.8 μM sodium arachidonate to the endothelial cells in culture increased the basal level of PGI₂ fourfold. Treating these cells with 10 nM of Factor Xa before adding fatty acid inhibited the production of the autacoid by nearly 50%. Factor Xa itself had no effect on the synthesis of PGI₂.

Factor V Antigen In Human Endothelial Cells

The interaction of Factor Xa with platelets is mediated through the binding of the protease to Factor Va on the platelet surface and Factor Va acts as the receptor for this binding. To determine whether the inhibition of PGI₂ synthesis by Factor Xa is due to the binding of the protease to endothelial cell Factor Va, we tried to ascertain if Factor Va was present in these cells. The competitive ELISA method showed that these cultured umbilical vein endothelial cells contained approximately 1.4 μg of Factor V antigen per 10⁷ cells on the first day after confluence (Figure 3). The level of Factor V antigen increased to 2.2 μg/10⁷/cell on the second day after confluence and declined to the basal level during the next 3 days. There was no appreciable antigen in the medium removed from these cultures. Thus, Factor V was not released by the cells into the medium. Bovine Factor V antigen in the fetal calf serum added to the culture medium (10%) crossreacted to less than 0.1% of human serum with the rabbit anti-Factor V antibody used in the ELISA. These results strongly suggest that the Factor V antigen was synthesized de novo in the cultured endothelial cells, but they do not pinpoint the intracellular site of Factor V.

Effect of Anti-Factor V Antibody on the Inhibition of PGI₂ Synthesis by Factor Xa

The presence of Factor V molecules in these endothelial cells might indicate that the interaction of Factor Xa with these cells (leading to the inhibition of PGI₂ synthesis) could be mediated through the interaction of Factor Xa with Factor Va molecules bound to the cell surface. If the inhibitory effect of Factor Xa is propagated through the above pathway where the surface Factor Va molecules serve as the receptors for Factor Xa interaction, it is possible that adding an antibody against Factor Va to the cell culture would counteract Factor Xa's inhibitory effect. The essential role of Factor V for Factor Xa's inhibition of PGI₂ formation was shown by treating the endothelial cells with a human monoclonal anti-Factor V antibody before adding Factor Xa (Figure 4).

Figure 3. Factor V antigen levels in human endothelial cells in culture. Factor V antigen levels were assayed by a competitive enzyme-linked immunosorbent assay method in the endothelial cell extract and in the medium in which the cells were grown. Because the amounts of Factor V antigen released from the cells into the medium were low, 10 ml of the medium was concentrated to 1 ml by ultrafiltration before the assay of the antigen. Each point is the means ± SEM of three experiments.

Figure 4. Reversal of Factor Xa-mediated inhibitor of PGI₂ formation in endothelial cells by human monoclonal anti-Factor V antibody. Human umbilical vein endothelial cells in tissue culture were treated with: 1) thrombin, 2) thrombin and Factor Xa, or 3) thrombin, Factor Xa, and the anti-Factor V antibody indicated. The experimental details are described in the Methods section. Each bar represents the means ± SEM of three experiments. The amounts of PGI₂ formed is expressed in per minute of incubation.
When the endothelial cells were incubated with anti-Factor V antibody (50 \( \mu \text{g/ml} \)) for 30 minutes at 37° C before the adding of Factor Xa (20 nM) and thrombin (24 nM), there was no inhibition of PGI\(_2\) synthesis by Factor Xa. The anti-factor V antibody itself had no effect on thrombin-stimulated PGI\(_2\) production. Neither Factor Xa nor anti-Factor V antibody had any effect on the basal level of formation of PGI\(_2\) in the endothelial cells. The countervailing effect of Factor V antibody on Factor Xa's inhibitory action was not a generalized protective property of immunoglobin. Normal human IgG (100 \( \mu \text{g/ml} \)) had no effect on the inhibiting effect of Factor Xa in endothelial cells under identical conditions.

**Discussion**

Factor Xa, which is generated by the activation of Factor X by both intrinsic and extrinsic pathways, is crucially important in blood coagulation.\(^{10-13}\) We have recently demonstrated\(^{20}\) that this important procoagulant enzyme is also a potent inhibitor of thromboxane \(_2\) synthesis in platelets. We show in the present study that this coagulation factor also has an inhibitory effect on the synthesis of a major prostanoid in endothelial cells.

Our studies also show for the first time that human endothelial cells contain Factor V molecules that seemed to act as the receptors for Factor Xa interaction. Several other observations are consistent with the presence and the important role of Factor V in endothelial cells. Bovine aortic endothelial cells have been shown\(^{23}\) to synthesize Factor V. Prothrombin conversion by Factor Xa is accelerated on the surface of bovine endothelial cells,\(^{24}\) and the process is blocked with an antibody against bovine Factor V, which suggests the presence of Factor V in the system. Preliminary studies\(^{25}\) indicate that Factor Va binds to endothelial cells and accelerates the activation of protein C. This reaction is blocked by the same human monoclonal antibody used in this study. The subcellular localization of Factor V in endothelial cells is unclear. We found no secretion into the medium under normal culture conditions, in agreement with Wilson et al.\(^{20}\) This finding does not detract from the putative synthesis of Factor V by endothelial cells. The countervailing effect of the Factor V antibody on the inhibition of PGI\(_2\) synthesis by Factor Xa in these cells indicated that the cell surface Factor Va molecules are receptors of the serine protease and that the formation of the Factor Va-Factor Xa complex on the cell surface is essential for prostaglandin synthesis.

The interaction of Factor Xa with the endothelial cells which down-regulates the formation of PGI\(_2\) could counteract thrombin's stimulatory effect. Since Factor Xa forms before thrombin, the endothelial cells might be conditioned not to respond to the stimulatory effect of thrombin. The inhibitory effect of Factor Xa might facilitate the formation of a hemostatic plug in vivo because the excessive formation of PGI\(_2\) would result in the inhibition of platelet aggregation. Factor Xa is a proteolytic enzyme, and as such the inhibitory action of the polypeptide on thrombin-stimulated PGI\(_2\) synthesis might be the result of thrombin proteolysis. This conclusion is not valid for several reasons. First, the procoagulant not only inhibited thrombin-stimulated PGI\(_2\) formation, but also inhibited the synthesis of PGI\(_2\) from sodium arachidonate by the endothelial cells. The inhibition of PGI\(_2\) synthesis stimulated by arachidonic acid by Factor Xa cannot be related to the proteolytic action of the enzyme. These results also indicate that the inhibitory effect of Factor Xa was not due to the interference of the ligand-receptor interaction. Second, reversal of the inhibitory effect of Factor Xa by the Factor V antibody would have been impossible if thrombin had been destroyed by Factor Xa's proteolytic action. Finally, investigators have reported\(^{13}\) that thrombin is not further cleaved by the enzymes of the blood coagulation cascade.

Human plasma contains approximately 100 nM of Factor X under normal conditions.\(^{20}\) Since the presence of 2 nM of Factor Xa inhibited about 60% of the PGI\(_2\) formation stimulated by thrombin, it is possible that the activation of only 2% of the total Factor X during the blood coagulation process might effectively down-regulate the synthesis of the prostanoid in the endothelial cells. The inhibitory effect of Factor Xa, a proteolytic enzyme, on the PGI\(_2\) synthesis in endothelial cells is not without precedent. Purified platelet calcium-activated cysteine protease also inhibits PGI\(_2\) synthesis in endothelial cells.\(^{27}\)

We have shown previously\(^{20}\) that Factor Xa inhibits thromboxane \(_2\) (TXA\(_2\)) synthesis in platelets. The present study indicates that this procoagulant also may control the PGI\(_2\) synthesis in endothelial cells. Since TXA\(_2\) is a potent vasoconstrictor and platelet-aggregating agent, and PGI\(_2\) is a vasodilator and inhibitor platelet aggregation, it is conceivable that overproduction of either prostanoid would interfere with the normal hemostatic mechanism. The generation of Factor Xa during the blood coagulation process cleaves prothrombin to thrombin through the formation of the prothrombinase complex, and it also might control the synthesis of PGI\(_2\) and TXA\(_2\) to maintain equilibrium.

**References**

5. Marcus AJ, Waksler BB, Jaffee E. Enzymatic conversion of...
INHIBITION OF PROSTACYCLIN FORMATION

prostaglandin endoperoxide \( \text{H}_2 \) and arachidonic acid to prostacyclin by cultured human endothelial cells. J Biol Chem 1978;253:7138-7141


11. Lundblad RL, Davie EW. The activation of Stuart factor (factor X) by activated antihemophilic factor (activated factor VIII). Biochemistry 1965;4:113-120

12. Esnouf MP, Williams WJ. The isolation and purification of a bovine plasma protein which is a substrate for the coagulation fraction of Russell’s viper venom. Biochem J 1962;84:62-71


14. Gimbrone MA. Culture of vascular endothelium. Prog Hemo-


Index Terms: endothelial cells • prostacyclin • Factor Xa • Factor V • arachidonic acid • Factor V antibody
Coagulant factor Xa inhibits prostacyclin formation in human endothelial cells. Role of factor V.
A K Sinha, A K Dutta-Roy, H C Chiu, G J Stewart and R W Colman

Arterioscler Thromb Vasc Biol. 1985;5:244-249
doi: 10.1161/01.ATV.5.3.244

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/5/3/244