Heparin Has an Inhibitory Effect on Endothelin-1 Synthesis and Release by Endothelial Cells

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We studied the inhibitory effects of heparin on basal and agonist-induced endothelin-1 biosynthesis and release from cultured bovine endothelial cells. Heparin dose-dependently and similarly inhibited endothelin-1 release, inositol trisphosphate production, and intracellular free Ca\(^{2+}\) levels stimulated by thrombin. Hirudin fragment had an inhibitory effect on thrombin-induced endothelin-1 release, whereas anti-thrombomodulin antibody had no effect. Heparin completely blocked phorbol ester–induced endothelin-1 release, whereas it had a partial inhibitory effect on endothelin-1 release stimulated by angiotensin and vasopressin. Northern blot analysis using complementary DNA for bovine preproendothelin-1 as a probe revealed that heparin reduced not only the basal but also the stimulated expression of preproendothelin-1 messenger RNA by thrombin and phorbol ester. These data suggest that heparin, in addition to its antithrombin effect, has an inhibitory effect on the biosynthesis and release of endothelin-1, possibly by inhibiting protein kinase C–dependent pathway.

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KEY WORDS • endothelins • thrombin • heparin • protein kinase C • RNA, messenger • endothelium

Endothelin-1 (ET-1) is a potent vasoconstrictor/pressor peptide initially isolated from the supernatant of cultured porcine endothelial cells.\(^1\) The induction of preproET-1 messenger RNA (mRNA) in endothelial cells is augmented by several agents, such as Ca\(^{2+}\) ionophore,\(^2\) phorbol ester,\(^2\) transforming growth factor-β,\(^3\) and cytokines.\(^4\) We have recently shown that the vasoconstrictor hormones angiotensin II (Ang II) and arginine vasopressin (AVP) induce preproET-1 mRNA expression in cultured bovine carotid artery endothelial (Bcae) cells\(^5\) and stimulate ET-1 release through receptor-mediated mobilization of intracellular free Ca\(^{2+}\) and activation of protein kinase C.\(^6\) Thrombin is also known to be a potent inducer of preproET-1 mRNA expression,\(^1\) although the cellular mechanism of its action has not been elucidated.

Thrombin, a key enzyme in the initiation of the coagulation process, is known to have at least two types of endothelial cell surface receptors: thrombomodulin, which acts as a cell surface cofactor to activate protein C,\(^7\) and a functional thrombin receptor coupled to G proteins.\(^8\) Heparin is a potent thrombin inhibitor with anticoagulant action mainly mediated by activation of two serine protease inhibitors (serpines), antithrombin III\(^9\) and heparin cofactor II.\(^10\) However, it has been shown that heparin can also inhibit conversion of fibrinogen into fibrin by direct interaction with anion-binding exosite of thrombin.\(^11\) Heparin also has a variety of inhibitory actions on vascular smooth muscle cells, including inhibition of cell proliferation and migration,\(^12,13\) inhibition of proto-oncogene (c-fos, c-myc) induction,\(^14\) and inhibition of collagenase\(^15\) and tissue-type plasminogen activator\(^16\) gene expression. Therefore, the present study was designed to examine the effects of heparin on the basal and stimulated biosynthesis and release of ET-1 by thrombin and other agonists.

Methods

Compounds

The following drugs were used: thrombin (Midori-Juji Co. Ltd., Osaka, Japan); heparin (Novo-Nordisk A/S, Gentofte, Denmark); [Tyr\(^{63}(SO_{3}H)\)]-hirudin fragment (54–65),\(^17\) Ang II, AVP, 12-O-tetradecanoylphorbol 13-acetate (TPA), indomethacin, and N\(^{6}\)-monomethyl-L-arginine (L-NMMA) (Sigma Chemical Co., St. Louis, Mo.); Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, Irvine, Scotland); fetal bovine serum (Cell Culture Laboratories, Cleveland, Ohio); \(N\)-[2-hydroxyethyl]piperazine-N-'\(2\)-ethanesulfonic acid] (HEPES), fura-2 acetoxyethyl ester (fura-2-AM), and EGTA (Dojink Chemicals, Kumamoto, Japan); \(^{125}\)I-ET-1 (Amersham Japan, Tokyo); and deoxycytidine 5'-[\(^{32}\)P] triphosphate (DuPont NEN Research Products, Boston). Polyclonal anti-thrombomodulin antibody was gen-
FIGURE 1. Line graphs show effect of heparin (panel A) and hirudin fragment (panel B) on endothelin-1-like immunoreactivity release from cultured bovine carotid artery endothelial cells. Cells were incubated with (●) or without (○) thrombin (2 units/mL) in the absence and presence of various doses of heparin (0.1–10 units/mL) or hirudin fragment (0.1–100 ng/mL) for 4 hours. Each point is the mean of six experiments; bar shows SEM. *Statistically significant difference (p<0.05) from control.

Cell Culture and Incubation

BCAE cells were prepared and cultured in DMEM containing 10% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere of 95% air-5% CO₂ as previously described. Cells harvested between the ninth and 12th passages were used in the experiments. To study the release of ET-1, confluent monolayer cells (5×10⁵) were usually preincubated in serum-free DMEM over 24 hours and then were placed in 1 mL fresh serum-free DMEM with agents and incubated for 4 hours unless otherwise specified.

Radioimmunoassay of Endothelin-1

ET-1-like immunoreactivity (ET-1-LI) in medium was measured by a specific radioimmunoassay for ET-1 using rabbit anti-ET-1 serum as previously described. The antibody has full cross-reactivity with ET-1 (100%), endothelin-2 (200%), and endothelin-3 (100%) but none with big ET-1. The sensitivity of the ET-1 radioimmunoassay was 1 fmol per tube, and the 50% intercept was 14 fmol per tube. The intra-assay and inter assay variations were 3.2% (n=6) and 8.6% (n=5), respectively.

Determination of Inositol 1,4,5-Trisphosphate

Confluent BCAE monolayers (10⁶ cells) in 12-well dishes were incubated with 20 mM LiCl at 37°C for 15 seconds; the incubation was terminated by the addition of ice-cold 10% perchloric acid. Perchloric acid extracts were neutralized with 1.54 M KOH/75 mM HEPES solution, and inositol 1,4,5-trisphosphate (IP₃) was determined by a competitive protein binding assay kit (DuPont NEN Research Products).

Determination of Intracellular Free Ca²⁺ Concentration

Confluent BCAE cells were trypsinized and incubated with 4 µM fura 2-AM at 37°C for 20 minutes in HEPES-buffered physiological salt solution as previously reported. The Ca²⁺-fura-2 fluorescence of the suspended cells (5×10⁶ cells/mL) was measured by a spectrofluorometer (CAF-100, JASCO Co. Ltd., Tokyo) using excitation of 340 and 380 nm and emission of 500 nm. Values of intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) were calculated according to the method of Grynkiewicz et al with the following formula:

\[ [Ca^{2+}]_i = K_d \times \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \times \frac{380_{\text{min}}}{380_{\text{max}}} \]

assuming that the K_d for the fura-2/Ca²⁺ complex is 224 nM at 37°C, R represents the ratio of fluorescence of the sample at 340 and 380 nm, and R_{max} and R_{min} are the ratios at maximal fluorescence with 10% Triton X-100 and minimal fluorescence with 15 mM EGTA, respectively.
Inhibition of Endothelin-1 Synthesis by Heparin

**FIGURE 2.** Bar graph shows effect of anti-thrombomodulin antibody (Anti TM Ab) on thrombin-induced endothelin-1-like immunoreactivity release. Bovine carotid artery endothelial cells were incubated with (●) or without (○) thrombin (2 units/mL) in the absence and presence of anti-thrombomodulin antibody (50 μg/mL) for 4 hours. Each column is the mean of three experiments; bar shows SEM.

**Northern Blot Analysis**

Confluent BCAE monolayers (3×10⁶ cells) in 60-mm culture dishes were incubated in 2 mL fresh serum-free DMEM with or without agonist and heparin for 30 minutes. Total RNAs from BCAE cells were then extracted with selective precipitation in 3 M LiCl/6 M urea, and cellular RNAs (5 μg per lane) were separated by formaldehyde/1.1% agarose gel electrophoresis and transferred to a MagnaGraph nylon membrane (Micron Separations Inc., Westboro, Mass.). After ultraviolet wave cross-linking, RNA immobilized on the membrane was hybridized with bovine preproET-1 complementary DNA (cDNA)³ as a probe in the presence of 50% formamide at 42°C over 16 hours. The probe was labeled with deoxycytidine 5′-[³²P]triphosphate (111 TBq/mmol) by the random-primed labeling method. The membrane was washed finally in 0.1×SSPE (15 mM NaCl, 1 mM NaH₂PO₄, 0.1 mM EDTA)/0.5% sodium dodecyl sulfate at 50°C and autoradiographed with an intensifying screen at −80°C for 24 hours.

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical analysis was performed by unpaired Student’s t test.

**FIGURE 3.** Line graph shows effect of heparin on thrombin-induced inositol 1,4,5-trisphosphate (IP₃) formation in cultured bovine carotid artery endothelial cells. Cells were incubated with (●) or without (○) thrombin (2 units/mL) in the absence and presence of various doses (0.5–10 units/mL) of heparin for 15 seconds. Each point is the mean of three experiments; bar shows SEM. *Statistically significant difference (p<0.05) from control.

**Results**

Thrombin time-dependently (4–24 hours) and dose-dependently (0.2–20 units/mL) stimulated release of ET-1-LI; the maximum effect was induced with 2 units/mL after a 24-hour incubation (data not shown). As shown in Figure 1, heparin (0.1–10 units/mL) and hirudin fragment (0.1–100 ng/mL) dose-dependently decreased ET-1-LI release stimulated by thrombin (2 units/mL); heparin in a maximal dose (10 units/mL) decreased thrombin-induced ET-1-LI release by 75%, and hirudin fragment (10–100 ng/mL) completely blocked the stimulatory effect to basal levels. Heparin also reduced the basal ET-1-LI release at higher doses (5–10 units/mL), whereas hirudin fragment did not. Anti-thrombomodulin antibody in a dose (50 μg/mL) sufficient to block thrombomodulin activity had no effect on the thrombin-induced ET-1-LI release (Figure 2).

Thrombin also dose-dependently (0.2–20 units/mL) induced immediate and transient formation of IP₃ and increase in [Ca²⁺], both of which peaked at 15 seconds and returned to prestimulated levels by 60 seconds.
FIGURE 4. Typical tracings from representative experiments show changes of intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in cultured bovine carotid artery endothelial cells. Cell suspensions loaded with fura-2 were challenged with thrombin (2 units/mL) alone (top tracing) and after pretreatment with heparin (10 units/mL) (bottom tracing). (data not shown). The thrombin-induced IP$_3$ formation was inhibited by heparin in a dose-dependent fashion (0.5-5 units/mL) (Figure 3). Heparin also reduced the thrombin-induced [Ca$^{2+}$]$_i$ increase (Figure 4).

Ang II (10$^{-6}$ M), AVP (10$^{-6}$ M), and TPA (10$^{-6}$ M) significantly stimulated ET-1-LI release (Figure 5) ($p<0.05$). In the presence of heparin, TPA-induced ET-1-LI release was completely inhibited, whereas ET-1-LI release stimulated by Ang II and AVP was partially, but significantly, decreased ($p<0.05$). Indomethacin (10$^{-6}$ M) and L-NMMA (2×10$^{-4}$ M) failed to affect the basal release or heparin-induced decrease in ET-1-LI release (data not shown).

Northern blot analysis using the cloned bovine preproET-1 cDNA as a probe revealed that the expression of preproET-1 mRNA (2.3 kb) was rapidly induced (within 15 minutes) by thrombin and TPA, reaching a peak by 30-60 minutes (data not shown). As shown in Figure 6, preproET-1 mRNA expression induced by thrombin (10 units/mL) and TPA (10$^{-6}$ M) was completely inhibited by heparin (20 units/mL). Heparin also reduced the basal expression of preproET-1 mRNA.

Discussion

Thrombin is a member of a serine protease family involved in coagulation pathways, such as activation of coagulation factors (V, VIII, and XIII) and conversion of fibrinogen to fibrin. Thrombin has a membrane-bound receptor on vascular endothelial cells, termed thrombomodulin, that acts as a cell surface cofactor to activate vitamin K-dependent protein C, thereby inactivating factors Va and VIIIa and subsequent inhibition of factor Xa in a negative feedback fashion. Based on the failure of the anti-thrombomodulin antibody to block the thrombin-induced ET-1-LI release, it is suggested that thrombomodulin is not involved in the mechanism of thrombin-mediated ET-1 release from endothelial cells.

The present study clearly shows that thrombin induces immediate and dose-dependent effects on IP$_3$ formation and [Ca$^{2+}$]$_i$ increase. These data are compatible with those of a previous study showing that thrombin induces phospholipase C-mediated phosphoinositide breakdown in human endothelial cells. The present study also shows that thrombin induces immediate expression of preproET-1 mRNA and subsequently releases ET-1-LI from BCAE cells. These data are also consistent with those previously reported.

Recently, a functional thrombin receptor has been cloned from human megakaryocytes; the thrombin receptor has seven transmembrane domains common to the G protein–coupled receptor superfamily. Thrombin may cleave the Arg$^{4'}$-Ser$_{42}$ bond of the amino terminal extracellular binding domain of the receptor, which is critical for its activation. Functional thrombin receptors

FIGURE 5. Bar graph shows effect of heparin on endothelin-1–like immunoreactivity release from bovine carotid artery endothelial cells. Cells were incubated with angiotensin II (Ang II) (10$^{-6}$ M), arginine vasopressin (AVP) (10$^{-6}$ M), and 12-O-tetradecanoylphorbol 13-acetate (TPA) (10$^{-6}$ M) in the absence (open column) and presence (shaded column) of heparin (10 units/mL) for 4 hours. Each column is the mean of six experiments; bar shows SEM.
FIGURE 6. Northern blot shows preproendothelin-1 (preproET-1) messenger RNA in cultured bovine carotid artery endothelial cells. Cells were incubated with thrombin (10 units/mL) (left blots) and 12-O-tetradecanoylphorbol 13-acetate (TPA) (10⁻⁶ M) (right blots) in the absence and presence of heparin (20 units/mL) for 30 minutes. Total cellular RNAs (5 µg per lane) were subjected to Northern blot analysis using the cloned bovine preproET-1 complementary DNA as a probe (top blots). Ribosomal RNA (rRNA) bands are shown as internal standard markers (bottom blots). Left lanes: 1, control; 2, heparin; 3, thrombin; 4, thrombin plus heparin. Right lanes: 1, control; 2, TPA; 3, thrombin plus heparin.

have been detected in human platelets and vascular endothelial cells by polymerase chain reaction analysis. Thus, it is suggested that thrombin, after binding to its functional receptor, stimulates phosphoinositide breakdown to induce preproET-1 mRNA. We have recently shown that Ang II and AVP induce preproET-1 mRNA expression and stimulate ET-1 release, possibly through activation of protein kinase C and mobilization of intracellular free Ca²⁺ resulting from receptor-mediated phosphoinositide breakdown. Taken together, these results suggest that the mechanism by which thrombin induces preproET-1 mRNA expression and subsequent ET-1 release may be similar to that by both Ang II and AVP.

Heparin is a proteoglycan produced by mast cells and acts as an anticoagulant, mainly by accelerating the inhibitory action of antithrombin III and heparin cofactor II in plasma. In this study, heparin has been shown to be effective in inhibiting thrombin-induced IP₃ formation, [Ca²⁺], increase, preproET-1 mRNA expression, and subsequent ET-1-LI release under a serum-free condition. It has been demonstrated that heparin, like anionic polymers, could potentially inhibit α-thrombin in the conversion of fibrinogen into fibrin by its direct interaction with anion-binding exosite of thrombin composed of clustered arginines and lysines. This region, although independent of the catalytic site, interacts with heparin, heparan sulfate, dermatan sulfate, dextran sulfate, and more specifically with hirudin. In this study, we have shown that hirudin fragment, which is a more potent and specific thrombin inhibitor than heparin, completely blocks the thrombin-induced ET-1-LI release from endothelial cells. Our data coincide with those of a previous study showing that hirudin inhibits the thrombin-induced, but not the spontaneous, release of ET-1 from intact porcine aorta in vitro. Taken together, these results show that heparin, like hirudin, may directly inhibit thrombin binding to its functional receptor by interacting with anion-binding exosite of thrombin to block phospholipase C-mediated phosphoinositide breakdown and [Ca²⁺] increase, thereby leading to the inhibition of the synthesis and release of ET-1.

The mechanism by which heparin, but not hirudin, decreases basal synthesis and release of ET-1 remains unknown. Heparin may exert its effects through other endothelium-derived relaxing substances, such as prostacyclin and endothelium-derived relaxing factor, both of which have been shown to inhibit ET-1 release. However, the involvement of prostacyclin and endothelium-derived relaxing factor in the inhibitory effect of heparin on ET-1 release seems unlikely because neither indomethacin nor L-NMMA exerts any effects on the basal or heparin-induced decrease in ET-1-LI release. Endothelial cells produce heparin-binding growth factors, presently known as acidic (a) and basic (b) fibroblast growth factors (FGFs). It has been shown that, in the presence of heparin, aFGF decreases prostacyclin synthesis in human endothelial cells and bFGF inhibits endothelial cell proliferation. Because FGFs induce receptor-mediated phosphoinositide breakdown and [Ca²⁺], increase in endothelial cells, it is possible to speculate that exogenous heparin in higher doses may inhibit phosphoinositide breakdown induced by endogenous FGFs or other yet uncharacterized factors from endothelial cells, thereby leading to a decrease in basal synthesis and release of ET-1.

The present study has further shown that heparin inhibits ET-1-LI release stimulated by Ang II, AVP, and TPA. It has been shown that heparin has a wide variety of inhibitory effects on vascular smooth muscle cells, including inhibition of cell proliferation and migration, inhibition of induction of proto-oncogenes (c-fos, c-myc) by serum and phorbol esters, and inhibition of nitric oxide synthesis. These observations suggest that heparin may have potential therapeutic applications in the treatment of vascular disease.
have been detected in human platelets and vascular endothelial cells by polymerase chain reaction analysis.\textsuperscript{8} Thus, it is suggested that thrombin, after binding to its functional receptor, stimulates phosphoinositide breakdown to induce preproET-1 mRNA. We have recently shown that Ang II and AVP induce preproET-1 mRNA expression\textsuperscript{5} and stimulate ET-1 release, possibly through activation of protein kinase C and mobilization of intracellular free Ca\textsuperscript{2+} resulting from receptor-mediated phosphoinositide breakdown.\textsuperscript{6} Taken together, these results suggest that the mechanism by which thrombin induces preproET-1 mRNA expression and subsequent ET-1 release may be similar to that by both Ang II and AVP.

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bition of gene expression of collagenase and tissue-type plasminogen activator. It has been suggested that these inhibitory actions of heparin are mediated by its selective blockade on the protein kinase C-dependent pathway. Furthermore, heparin has been shown to inhibit cyclic AMP–dependent protein kinase and tyrosine kinase other than protein kinase C. In fact, our study has shown that heparin partially inhibits Ang II– and AVP-induced ET-1 release, whereas heparin completely blocks TPA-induced preproET-1 gene expression as well as ET-1-LI release. Because it is well recognized that Ang II and AVP have common signal-transduction systems, consisting of IP3-mediated intracellular Ca2+ mobilization and diacylglycerol-activated protein kinase C pathways, it seems reasonable to speculate that heparin may preferentially inhibit the protein kinase C–dependent pathway over the Ca2+-dependent pathway in endothelial cells.

The inhibitory actions of heparin on basal and agonist-induced ET-1 synthesis and release provide a potential therapeutic implication for its use in preventing the overexpression of the ET-1 gene in the vascular lesions associated with thromboembolic disease, hypertension, and atherosclerosis.

References


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