Effects of Amrinone on Thrombin-Induced Platelet-Derived Growth Factor-Like Protein Secretion from Endothelial Cells

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ABSTRACT. Endothelial cells (EC) secrete platelet-derived growth factor (PDGF)-like protein, which is a potent mitogen to smooth muscle and connective tissue cells. The purpose of this study was to determine if amrinone, a phosphodiesterase inhibitor, could inhibit PDGF-like protein secretion on the basis of its ability to increase cAMP. Human umbilical artery endothelial cells (HUAEC) ($n = 7$) were preincubated for 4 h with amrinone ($10 \mu\text{g/mL}$) before incubation with thrombin (10 IU/mL) and amrinone ($10 \mu\text{g/mL}$) for 18 h. The supernatant was then assayed for the presence of both PDGF-like protein by using a competitive ^{125}I -PDGF radioreceptor inhibition assay, and cAMP by using an RIA. Thrombin-induced PDGF-like protein secretion from HUAEC was significantly inhibited by amrinone ($7.8 \pm 1.6 \text{ fmol}/10^6 \text{ EC}$) when compared with thrombin alone ($12.1 \pm 2.4 \text{ fmol}/10^6 \text{ EC}$) ($p < 0.05$). Amrinone alone had no effect on baseline PDGF-like protein secretion. Amrinone inhibition of thrombin-induced PDGF-like protein secretion was comparable whether amrinone was added to HUAEC 4 or 0 h before thrombin, and it was dose dependent with a maximal inhibition of 82.7% by amrinone ($160 \mu\text{g/mL}$). In contrast, IL-1 α ($10 \mu\text{g/mL}$) and tumor necrosis factor (100 ng/mL) induced less secretion of PDGF-like protein from HUAEC, and this secretion was not inhibited by amrinone. Amrinone ($10 \mu\text{g/mL}$) significantly increased secretion of cAMP from HUAEC from a baseline value of $6.4 \pm 0.4 \text{ pmol}/10^6 \text{ EC}$ to $10.6 \pm 0.1 \text{ pmol}/10^6 \text{ EC}$ ($p < 0.01$). We conclude that amrinone inhibits thrombin-induced PDGF-like protein secretion from HUAEC. This ability of amrinone to inhibit thrombin-induced PDGF-like protein secretion from EC is associated with an increase in secretion of cAMP from EC, suggesting that this inhibition is associated with, or mediated through, an increased production of cAMP. (*Pediatr Res* 30: 351–354, 1991)

Abbreviations

EC, endothelial cell
ECCM, endothelial cell conditioned medium
HUAEC, human umbilical artery endothelial cell
PDE, phosphodiesterase
PDGF, platelet-derived growth factor
PDS, plasma-derived serum
TNF, tumor necrosis factor

Pulmonary vascular hypertension, atherosclerosis, and post-transplant accelerated graft arteriosclerosis are major causes of morbidity and mortality in both adults and children. A major component of the pathophysiology of all of these processes is smooth muscle and connective tissue proliferation (1–4). PDGF is a serum mitogen that causes proliferation of smooth muscle and connective tissue cells (4, 5). PDGF is found in human serum as a heterodimer of two polypeptide chains (PDGF-A and PDGF-B). PDGF has been implicated in a variety of disease processes by showing the presence of increased PDGF-B-type receptors on vascular smooth muscle cells in atherosclerotic plaques, rejection of transplanted kidneys, and chronic synovitis of rheumatoid arthritis (6). PDGF gene expression is also increased in human atherosclerotic plaques (7). PDGF-like protein is produced by EC, smooth muscle cells, activated macrophages, and mitogen-stimulated fibroblasts (5). Production of PDGF-like protein from cultured EC is stimulated by many factors, including thrombin, IL-1, and TNF (8, 9). Work from this laboratory has also shown that T cells are capable of inducing PDGF-like protein from umbilical artery EC (10). The agents currently known to inhibit release of PDGF-like protein from EC include modified LDL, basic fibroblast growth factor, and agents that increase cAMP (isoproterenol, norepinephrine, and forskolin) (11–13). PDE III inhibitors also increase cAMP by inhibition of cAMP degradation. Amrinone is a selective PDE III inhibitor with positive inotropic, chronotropic, and vasodilatory properties (14). We hypothesized that amrinone, through its ability to increase cAMP, may also have utility in regulating PDGF-like protein secretion from EC. The purpose of this study was to investigate the inhibitory potential of amrinone on thrombin-induced PDGF-like protein secretion from HUAEC.

MATERIALS AND METHODS

HUAEC culture. Umbilical cords were obtained on a daily basis from a busy labor and delivery department of a local hospital. HUAEC were cultured as previously described by our laboratory (15). Briefly, a 3.5 or 5 French umbilical vessel catheter was inserted into one of the umbilical arteries and the vessel was flushed with physiologic saline until free of blood, infused with 0.2% collagenase, and incubated for 20 min in 5% CO₂-95% air at 37°C. The EC aspirated from the cord were resuspended in complete EC medium: M199 containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, supplemented with L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 $\mu\text{g/mL}$), and 20% pooled human serum. The EC were then pooled from several cords and plated onto 0.2% gelatin-coated dishes. These cells reached tight confluence within 3 to 5 d. The medium was changed every 3rd d. All HUAEC used in this experiment were primary cultures. EC identity was verified by the characteristic "cobblestone" morphology under phase-contrast light microscopy and by demonstration of factor VIII-von Willebrand factor antigen by direct immunofluores-

Received October 8, 1990; accepted May 23, 1991.

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cence with fluorescein-conjugated anti-von Willebrand factor antibody (16).

3T3 cell culture. 3T3 cells generously provided by Dr. Martin Rechsteiner of the University of Utah Department of Biochemistry were grown in 75-cm² tissue culture flasks (Falcon Becton Dickinson Labware, Lincoln Park, NJ) and maintained in complete 3T3 cell medium containing 90% Dulbecco's modified Eagle medium (Sigma Chemical Co., St. Louis, MO) and 10% FCS (Hyclone, Logan, UT). Medium was changed twice weekly, and the cells were passed in subculture 1:3 upon reaching confluence. Samples of the cells were also frozen in liquid nitrogen for subsequent use.

Generation of ECCM. Because serum contains PDGF, all HUAEC monolayers were incubated for 4 h in 1% PDS medium before pre- and coincubations, which were also performed in EC medium containing 1% PDS. PDS was prepared as previously described (8). Whole blood from healthy, normal donors was drawn into precooled syringes with 3.8% sodium citrate, and cell-free plasma was then recalcified with 1 M CaCl₂ (1:50), followed by incubation at 37°C for 2 h and centrifugation at 25 000 × g at 4°C for 20 min. The PDS was then filtered (0.22 µm pore size), divided into samples, and stored at -70°C until use. To induce PDGF-like protein secretion, HUAEC were incubated with thrombin (10 IU/mL) for 18 h as previously described (10). HUAEC were also incubated with IL-1α (R & D Systems, Minneapolis, MN) (10 µg/mL) and TNF (generously provided by Cetus Corp., Emeryville, CA) (100 ng/mL). Amrinone (a generous gift of Winthrop Pharmaceuticals, New York City, NY) was added to the HUAEC either 4 or 0 h before the addition of thrombin. Because we detected no difference between 4- and 0-h preincubation of amrinone with thrombin, all studies with IL-1 and TNF were performed with amrinone simultaneously added to the HUAEC. After an 18-h incubation in 5% CO₂-95% air at 37°C, the supernatant from the HUAEC monolayers was removed, centrifuged at 1100 rpm for 10 min to remove all cellular debris, and then assayed immediately for the presence of PDGF-like protein by the assay described below. Background PDGF-like protein secretion was determined by assaying the supernatant from HUAEC incubated for 18 h in 1% PDS alone. The HUAEC were evaluated by phase-contrast light microscopy for the ability to exclude trypan blue to estimate cell death.

PDGF-like protein secretion from HUAEC. ECCM was assayed for the presence of PDGF-like protein by a ¹²⁵I-PDGF (Dupont, Boston, MA) competitive radioreceptor binding assay (17). Before the study, 24-well dishes of confluent 3T3 cells were incubated in serum-free 3T3 cell medium. The dishes were then placed in an ice tray, and the 3T3 cells were washed once with ice-cold binding medium [Dulbecco's modified Eagle medium containing 0.22% NaHCO₃ and BSA (2 mg/mL)]. Then, 400 µL of either serum-free 3T3 medium (control), 50 ng of unlabeled PDGF (to assess nonspecific binding), or various ECCM were added to each well, and the dishes were incubated at 4°C with gentle agitation (150 rpm) for 2 h. After this incubation, the medium on the cells was aspirated and discarded, and the cells were washed once with ice-cold binding medium. Each well then received 0.4 ng of ¹²⁵I-PDGF, and the dishes were incubated again at 4°C with gentle agitation (150 rpm) for 2 h. After this second incubation, the supernatant and two washes were collected and the 3T3 cells were lysed with 1% Triton X-100. The supernatant, wash, and cell lysate were counted separately in a Beckman 4000 gamma counter. The amount of PDGF-like protein present under experimental conditions in ECCM was determined by the percentage of inhibition of maximal ¹²⁵I-PDGF binding to the EC and was expressed as fmol/10⁶ cells of PDGF-like protein secreted. Maximal ¹²⁵I-PDGF binding was determined by ¹²⁵I-PDGF presence in the cell lysate fraction of 3T3 cells after a 2-h incubation with serum-free 3T3 medium (control). Nonspecific binding was determined by the percentage of binding of maximal ¹²⁵I-PDGF binding to 3T3 cells after a 2-

h incubation with a 100-fold excess of cold PDGF. Initial experiments were performed in triplicate, and dose-response experiments were performed in duplicate. To account for radioactive decay, a 1-ng sample of ¹²⁵I-PDGF was counted separately with each experiment to determine counts/ng of ¹²⁵I-PDGF.

Preliminary studies were done to determine binding characteristics of ¹²⁵I-PDGF to the 3T3 cells. Standard curves were obtained with ¹²⁵I-PDGF (0.05–0.4 ng/well) and 100-fold excess PDGF to determine amounts of specific and nonspecific binding. Nonspecific binding was 13.9 ± 1.8%.

cAMP secretion from HUAEC. Confluent HUAEC monolayers in 25-cm² flasks were incubated with 1% PDS (control) or amrinone (10 µg/mL) for 18 h at 37°C. After the incubation, the flasks were chilled on ice, the medium was aspirated from the cells, boiled for 5 min, and centrifuged, and the supernatant was frozen at -70°C for cAMP assay (18). Measurement of cAMP in supernatants of EC was performed by RIA, with ¹²⁵I-cAMP tracer (Amersham Corp., Arlington Heights, IL).

Statistical analysis. Comparisons between different experimental conditions were made using a paired *t* test. Values with *p* < 0.05 were considered statistically significant. All data is expressed as mean ± SEM.

RESULTS

PDGF-like protein secretion was significantly inhibited when HUAEC were preincubated with amrinone 4 h before coincubation with thrombin for 18 h (7.8 ± 1.6 fmol/10⁶ EC) compared with thrombin alone (12.1 ± 2.4 fmol/10⁶ EC) (*n* = 7) (Fig. 1). Background PDGF-like protein secretion was <1 fmol/10⁶ EC. Amrinone also had no effect on background PDGF-like protein secretion.

Amrinone inhibition of thrombin-induced PDGF-like protein secretion was comparable when amrinone was added to HUAEC either 4 or 0 h before thrombin. In the 4-h preincubation, amrinone inhibited thrombin-induced PDGF-like protein secretion by 35.5%, whereas, in the 0-h preincubation, amrinone inhibited thrombin-induced PDGF-like protein secretion by 34.7% (*n* = 8).

PDGF-like protein secretion from HUAEC coincubated with thrombin and amrinone (2.5, 10, 40, and 160 µg/mL) for 18 h was inhibited in a dose-dependent fashion (Fig. 2). Thrombin-induced PDGF-like protein secretion was inhibited 82.7% by 160 µg/mL of amrinone, 66.7% by 40 µg/mL of amrinone, 33.3% by 10 µg/mL of amrinone, and 14.7% by 2.5 µg/mL of amrinone. Studies were also carried out with 320 µg/mL of amrinone; however, postincubation examination of the HUAEC with trypan blue revealed them to be very shrunken with a significant amount of cell death. Postincubation trypan blue exam of HUAEC in all other studies showed the cells to be viable with minimal trypan blue uptake.

IL-1α induced secretion of about one half as much PDGF-like

Fig. 1. Inhibition of thrombin-induced PDGF-like protein by amrinone (7.8 ± 1.6 fmol/10⁶ EC) compared with thrombin alone (12.1 ± 2.4 fmol/10⁶ EC) (*, *p* < 0.05).

Fig. 2. Dose-response inhibition of thrombin-induced PDGF-like protein by amrinone.

Fig. 3. PDGF-like protein secretion induced by IL-1 (10 µg/mL) and TNF (100 ng/mL), demonstrating lack of inhibition of PDGF-like protein secretion by amrinone (10 µg/mL).

Fig. 4. cAMP production by HUAEC after control incubation with 1% PDS and after incubation with amrinone (10 µg/mL) (*, $p < 0.05$).

protein from HUAEC (6.0 ± 1.32 fmol/10⁶ EC) as thrombin, and this secretion was not significantly inhibited by simultaneous addition of 10 µg/mL of amrinone (5.2 ± 1.3 fmol/10⁶ EC) ($n = 8$) (Fig. 3). TNF induced an even smaller increase in PDGF-like protein secretion from HUAEC (2.3 ± 0.6 fmol/10⁶ EC) than was seen with thrombin ($n = 8$). Similarly, this degree of PDGF-like protein secretion was not inhibited by the simultaneous addition of amrinone (10 µg/mL).

Secretion of cAMP from HUAEC after incubation with amrinone for 18 h was significantly greater (10.6 ± 0.1 pmol/10⁶ EC) than control (6.4 ± 0.7 pmol/10⁶ EC) ($p < 0.05$) (Fig. 4).

DISCUSSION

EC secrete PDGF-like protein, which has been identified by biochemical and immunologic criteria (19) and by its ability to

competitively block binding of ¹²⁵I-PDGF to its specific cell surface receptor on a variety of different cell types (8). This protein is a potent mitogen for Swiss 3T3 fibroblasts and smooth muscle cells and is continuously secreted in low levels by healthy, unstimulated EC (8, 20). EC can be stimulated to secrete PDGF-like protein by stimuli such as thrombin, IL-1, and TNF. Thrombin is a serine protease generated at sites of vascular injury that (in addition to many other functions) induces cultured EC both to express the *c-sis* mRNA coding for the B chain of PDGF (21) and to release PDGF-like protein from EC into the culture medium (8). It is speculated that thrombin exerts at least some of its effect on PDGF-like protein by converting molecules present in EC that are not recognized by the PDGF receptor to PDGF-like protein, which is then released (8). The mechanisms of IL-1 and TNF on induction of PDGF-like protein secretion from EC are less clear.

This study shows that amrinone inhibits thrombin-induced PDGF-like protein secretion from HUAEC. This inhibition is dose dependent and does not require HUAEC preincubation with amrinone. Because amrinone increased secretion of cAMP from HUAEC at dosages that increased PDGF-like protein secretion, we hypothesize that amrinone inhibition is associated with, or mediated through, elevation of cAMP levels. Amrinone exerts its effect at the cellular level through inhibition of PDE III, which is found in cardiac muscle, vascular smooth muscle, and human platelets. Inhibition of PDE III leads to decreased degradation of cAMP, subsequent accumulation of cAMP, and enhanced intracellular calcium accumulation (14, 22–24). The concentrations of amrinone required to inhibit PDGF-like protein secretion in our study were very similar to those required to inhibit PDE activity in cultured heart cells from rats (22) and in soluble and particulate fractions of hearts in humans (24). This supports the previous finding that agents which increase cAMP accumulation block endothelial *c-sis* induction by thrombin (11).

The fact that amrinone had no effect on PDGF-like protein secretion from HUAEC induced by IL-1 or TNF is possibly because of the diminished ability of IL-1 or TNF to induce PDGF-like protein secretion from HUAEC when compared with thrombin. IL-1 is a multifunctional cytokine that has been shown to induce both PDGF-AA and PDGF-B chain (*c-sis*) secretion from EC by mitogenesis assays and Northern blot analysis (25, 26). Hajjar *et al.* (9) demonstrated the ability of TNF to increase PDGF secretion from cultured EC, also by using mitogenesis assays and Northern blot analysis. These methods are different than the radioreceptor inhibition assay used in this study and thus make it difficult to compare absolute quantities of PDGF-like protein secreted. However, it is possible that the effects of amrinone only become evident when larger quantities of PDGF-like protein are secreted, such as that induced by thrombin. It is also possible that different mechanisms of induction of PDGF-like protein among IL-1, TNF, and thrombin exist, and amrinone only inhibits the mechanism of thrombin-induced PDGF-like protein secretion.

Time-response studies were performed to determine whether EC need to be preincubated with amrinone before coincubation with thrombin to inhibit PDGF-like protein secretion. Previous studies with agents that directly increase cAMP have shown that simultaneous addition of these agents with thrombin to EC effectively inhibited *c-sis* expression (11). However, because calcium accumulation after amrinone administration takes up to 4 h (22), it was conceivable that simultaneous coincubation of amrinone with thrombin may be less effective in inhibition of PDGF-like protein secretion than 4-h preincubation of EC with amrinone. The fact that there was no difference between amrinone preincubation and simultaneous coincubation with thrombin suggests that the effectiveness of amrinone in blocking thrombin-induced PDGF-like protein secretion is not enhanced by high intracellular calcium concentrations before thrombin administration.

These findings suggest that amrinone may have clinical poten-

tial for treatment or prevention of disease processes associated with myointimal proliferation. Although catecholamines have previously been shown to inhibit thrombin-induced PDGF-like protein secretion from EC, long-term use causes tachyphylaxis with decreased number of β -receptors, uncoupling of the receptors from adenylate cyclase, and accelerated cAMP degradation (27). Because of these disadvantages, a medication such as amrinone may have potential for long-term PDGF-like protein inhibition. Oral amrinone has been used clinically in trials to treat chronic congestive heart failure as well as pulmonary hypertension in heart transplant patients awaiting transplantation (28–30). Clinical utility in infants may be limited by the apparent differential effects of amrinone on newborn and adult myocardium (31, 32). However, further work is needed to determine *in vivo* effects of amrinone on the progression of myointimal proliferation.

In conclusion, this study demonstrates that amrinone inhibits thrombin-induced PDGF-like protein secretion from HUAEC and that this inhibition is dose dependent. This suggests a potential clinical role of phosphodiesterase inhibitors such as amrinone in the prevention and treatment of disease processes associated with myointimal proliferation.

Acknowledgments. The authors thank the staff of Labor and Delivery at Cottonwood Hospital for their generous supply of umbilical cords and Kris Sjoblom for manuscript preparation.

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