Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3456–3459, May 1986
Medical Sciences

Protease and cyclooxygenase inhibitors synergistically prevent activation of human platelets

(cerebral ischemia/cardiovascular disease/antiplatelet therapy/platelet aggregation and secretion)

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Communicated by John Vane, December 31, 1985

ABSTRACT **Thrombin induces platelet aggregation and formation of a fibrin clot in platelet-rich plasma; leupeptin, a protease inhibitor, partially inhibits platelet aggregation, but it does not inhibit fibrin clot formation. Indomethacin does not inhibit either thrombin-induced platelet aggregation or fibrin clot formation. However, when the two drugs are given together, a synergistic inhibition of thrombin-induced platelet aggregation occurs, while fibrin clot formation remains unaffected. Thrombin-induced stimulation of the release of serotonin in washed human platelets is also synergistically inhibited by the combined actions of leupeptin and indomethacin. Thrombin and collagen, added simultaneously, induce full platelet aggregation and release of serotonin. Neither leupeptin nor indomethacin inhibits platelet responses elicited by both agonists; however, when leupeptin and indomethacin are given together, a synergistic inhibition of thrombin- and collagen-induced response is observed. These findings might be relevant in prophylaxis and treatment of thromboembolic disease.**

Antiplatelet drugs—aspirin, dipyridamole, and sulfinpyrazone—have been shown to inhibit thrombus formation in experimental animal models, presumably by preventing platelet activation at sites of vascular injury. Several clinical trials have tested the effectiveness of these drugs in situations where platelet phenomena and thrombus formation may play an important role in the pathogenesis of certain clinical disorders—e.g., stroke and myocardial infarction (1–5).

The overall results, however, have been equivocal. This has been attributed to the inhibition of vascular prostacyclin biosynthesis coincident with that of thromboxane A_2 by the doses of cyclooxygenase inhibitors used in many clinical trials (5–7) and to the inability of cyclooxygenase inhibitors to inhibit thrombin-induced platelet activation (1).

We have recently shown that leupeptin, a protease inhibitor, is able to block human platelet responses to thrombin and trypsin, while it does not inhibit serotonin release and aggregation induced by collagen (8). We have now investigated the ability of a combination of leupeptin and indomethacin to inhibit platelet aggregation and serotonin release induced by thrombin and by thrombin plus collagen.

MATERIALS AND METHODS

Materials. Leupeptin (synthetic acetyl-L-leucyl-L-leucyl-DL-argininal), indomethacin, and thrombin (from human plasma) were obtained from Sigma. Collagen (1 mg of suspended equine collagen fibrils per 1 ml of isotonic glucose solution) was obtained from Hormon-Chemie (Munich, F.R.G.).

Preparation and Labeling of Human Platelets with [^{3}H]Serotonin. Platelet samples were prepared from fresh human blood (200 ml) treated with 0.20 ml of anticoagulant ACD buffer (85 mM trisodium citrate/111 mM dextrose/71 mM citric acid). Donors had not received any medication in the previous 3 weeks. Platelet-rich plasma was obtained by centrifugation at 200 × *g* for 25 min. Samples (0.5 ml) of platelet-rich plasma were placed in aggregometer tubes and preincubated (while stirring) for 1 min at 37°C in a Chrono-Log (Haverton, PA) aggregometer. Then addition of leupeptin (10 μg/ml) and/or indomethacin (10–20 μM) for 1 min was followed by the addition of thrombin (0.5 unit/ml). Light transmission was recorded throughout the experiment and displayed on a chart recorder.

To obtain washed platelets, platelet-rich plasma was centrifuged at 200 × *g* for 15 min. This platelet pellet was resuspended in 10 ml of a modified Tyrode–Hepes buffer (134 mM NaCl/12 mM $NaHCO_3$/2.9 mM KCl/0.36 mM NaH_2PO_4/1 mM $MgCl_2$/5 mM Hepes/5 mM glucose, pH 7.4), and 10 ml of the platelet suspension was labeled with 50 μCi (1 Ci = 37 GBq) of [^{3}H]serotonin at 37°C for 90 min. The platelet pellet was then washed with 30 ml of buffer, and platelet concentration was adjusted to 8×10^8 per ml. Samples (0.5 ml) were placed in aggregometer tubes and preincubated (while stirring) for 1 min at 37°C in a Chrono-Log aggregometer. Then leupeptin (0.5–20 μg/ml) and/or indomethacin (1–20 μM) were added for 1 min, followed by thrombin (0.3 unit/ml) or thrombin (0.3 unit/ml) and collagen (20 μg/ml). Light transmission was recorded throughout the experiment and displayed on a chart recorder.

Measurement of [^{3}H]Serotonin. Samples (0.2 ml) of platelets labeled with [^{3}H]serotonin were taken from the aggregometer tubes. Those samples were immediately mixed with 0.2 ml of 6% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3). Platelets were then centrifuged for 1 min in a Beckman Microfuge B. After centrifugation, ^{3}H radioactivity in 0.2 ml of the supernatant (which corresponds to 0.1 ml of the original platelet suspension) was determined by liquid scintillation counting. Release of [^{3}H]serotonin in these supernatants was calculated as the percentage of total radioactivity of 0.1 ml of platelet suspension not treated. Radioactivity in the bathing fluid was always subtracted before performing these calculations.

RESULTS

Effect of Leupeptin and Indomethacin on Thrombin-Induced Platelet Aggregation in Platelet-Rich Plasma. Thrombin (0.5 unit/ml) induced platelet shape change and aggregation in platelet-rich plasma, as shown in Fig. 1*A* by a gradual increase in light transmission. After 30–35 sec, however, an abrupt decrease of light transmission in the recorder reflected formation of a fibrin clot. Leupeptin (10 μg/ml), added 1 min before thrombin (0.5 unit/ml), partially inhibited thrombin-

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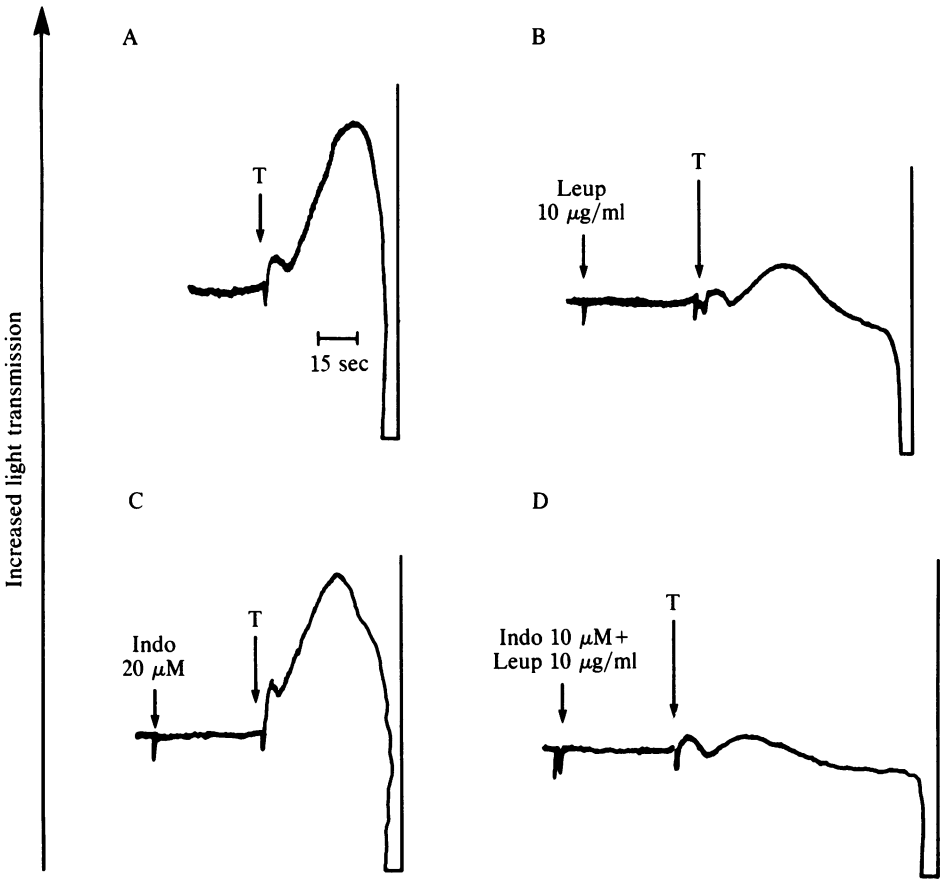

FIG. 1. Leupeptin and indomethacin synergistically inhibit aggregation induced by thrombin in platelet-rich plasma. Platelet-rich plasma (0.5 ml) was incubated with leupeptin (Leup; 10 μg/ml) and/or indomethacin (Indo; 10–20 μM) for 1 min (while stirring) in the aggregometer tube at 37°C. Thrombin (0.5 unit/ml) was then added (arrow labeled "T"); light transmission was recorded throughout the experiment to determine the extent of platelet aggregation (revealed by a gradual increase in light transmission) and the formation of a fibrin clot (revealed by an abrupt decrease in light transmission followed by a rapid increase).

induced platelet aggregation, but it did not inhibit fibrin clot formation (Fig. 1*B*). Indomethacin (up to 20 μM) added 1 min before thrombin (0.5 unit/ml) did not inhibit either platelet aggregation or fibrin clot formation (Fig. 1*C*). However, when leupeptin (10 μg/ml) and indomethacin (10 μM) were given together 1 min before thrombin (0.5 unit/ml), platelet aggregation was completely inhibited, while fibrin clot formation was unaffected (Fig. 1*D*).

Effect of Leupeptin and Indomethacin on Thrombin-Induced Serotonin Release in Washed Human Platelets. Thrombin (0.3 unit/ml) induced the release of approximately 95% of [^{3}H]serotonin in washed human platelets (Fig. 2). Leupeptin (0.5–20 μg/ml) added 1 min before thrombin (0.3 unit/ml) inhibited thrombin-induced serotonin release, and maximal inhibition occurred with leupeptin at 20 μg/ml (Fig. 2). Indomethacin (1 μM) added 1 min before thrombin (0.3 unit/ml) inhibited 45% of thrombin-induced serotonin release (Fig. 2). However, when both inhibitors were given together 1 min before thrombin, full inhibition of serotonin release occurred with leupeptin at only 2.5 μg/ml. This significant shift to lower concentrations of leupeptin for nearly total suppression of activity clearly demonstrates a synergistic pattern of inhibition (Fig. 2).

Effect of Leupeptin and Indomethacin on Thrombin/Collagen-Induced Aggregation in Washed Human Platelets. Simultaneous addition of thrombin (0.3 unit/ml) and collagen (20 μg/ml) induced maximal aggregation in washed human platelet (Fig. 3*A*). Leupeptin (up to 20 μg/ml) added 1 min before the thrombin and collagen did not inhibit platelet aggregation induced by both agonists (Fig. 3*B*). Similarly, indomethacin (up to 20 μM) added 1 min before thrombin and collagen did not inhibit platelet aggregation induced by both agonists (Fig.

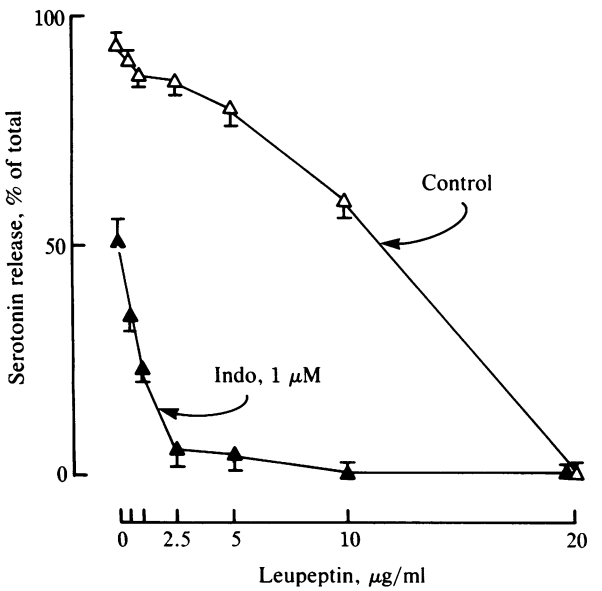

FIG. 2. Synergism of leupeptin and indomethacin on the inhibition of the release of serotonin induced by thrombin in human platelets. Washed human platelets (0.5 ml) labeled with [^{3}H]serotonin were incubated with different concentrations of leupeptin with (▲) and without (△) 1 μM indomethacin for 1 min (while stirring) in the aggregometer tube at 37°C. Thrombin (0.3 unit/ml) was then added for 1 min, and the release of [^{3}H]serotonin was measured. Control (△) indicates the action of thrombin in the absence of indomethacin. Results are the average of four experiments ± SEM. Total platelet radioactivity in 100-μl samples was 197,898 ± 1,989 (mean ± SEM; n = 4).

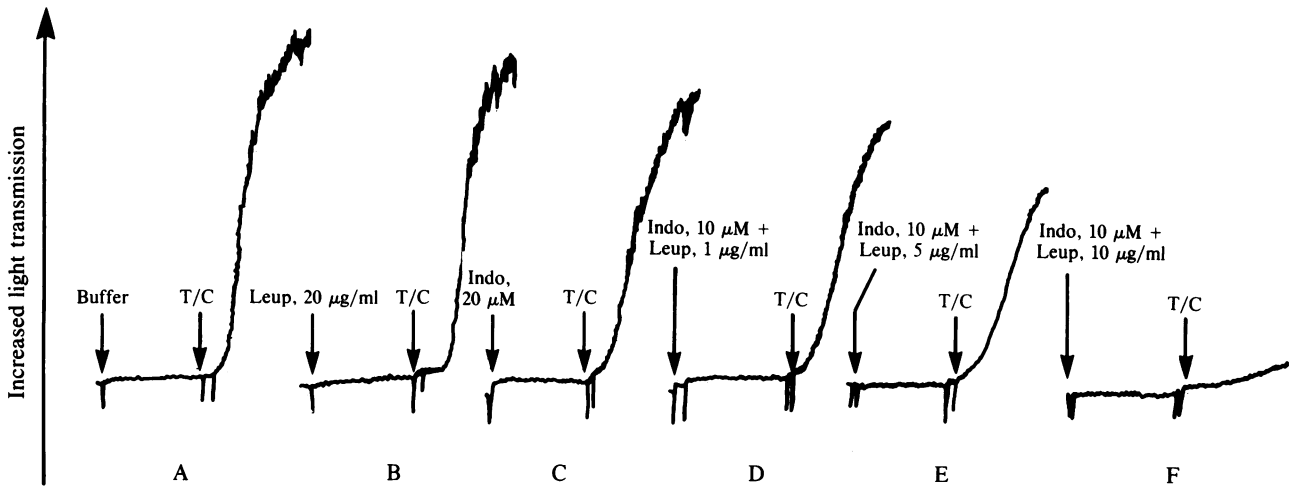

FIG. 3. Synergistic effect of leupeptin and indomethacin on human platelet aggregation induced by thrombin plus collagen. Washed human platelets (0.5 ml) were incubated with different concentrations of leupeptin (Leup) and/or indomethacin (Indo) for 1 min (while stirring) in the aggregometer tube at 37°C. Thrombin (0.3 unit/ml) and collagen (20 μg/ml) were then added for 1 min (arrows labeled "T/C"). Light transmission was recorded throughout the experiment to determine the extent of platelet aggregation.

3C). However, when leupeptin (1–10 μg/ml) and indomethacin (10 μM) were given together 1 min before thrombin and collagen, complete inhibition of platelet aggregation was observed. Maximal inhibition was observed with a combination of 10 μM indomethacin and leupeptin at 10 μg/ml (Figs. 3 *D*, *E*, and *F*).

Effect of Leupeptin and Indomethacin on Thrombin/Collagen-Induced Serotonin Release in Washed Human Platelets. Simultaneous addition of thrombin (0.3 unit/ml) and collagen (20 μg/ml) induced the release of approximately 98% of [^{3}H]serotonin in washed human platelets (Fig. 4). Leupeptin (up to 20 μg/ml) added 1 min before thrombin and collagen did not inhibit significantly the release of serotonin induced by both agonists (Fig. 4). Similarly, indomethacin (5–10 μM) added 1 min before thrombin and collagen only partially inhibited the release of serotonin induced by both agonists (Fig. 4). However, when leupeptin (0.5–20 μg/ml) and indomethacin (5–10 μM) were given together 1 min before thrombin and collagen, a dramatic synergistic inhibition of the effect of both agonists was evident (Fig. 4).

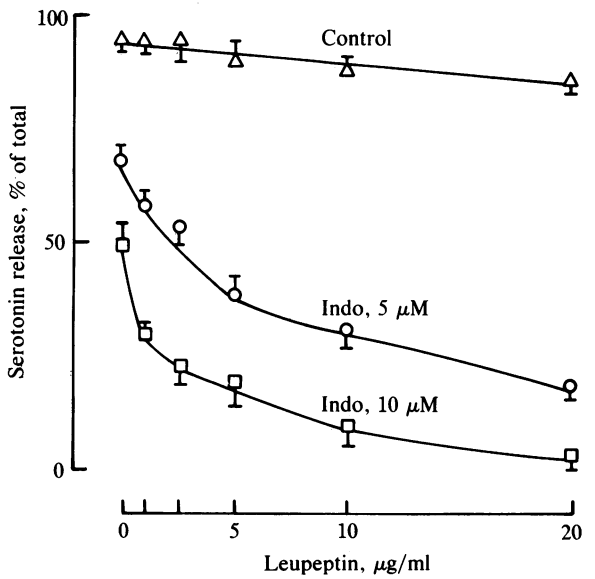

FIG. 4. Synergism of leupeptin and indomethacin on the inhibition of the release of serotonin induced by thrombin plus collagen in human platelets. Washed human platelets (0.5 ml) labeled with [^{3}H]serotonin were incubated with different concentrations of leupeptin and/or indomethacin (Indo) for 1 min (while stirring) in the aggregometer tube at 37°C. Thrombin (0.3 unit/ml) and collagen (20 μg/ml) were then added for 1 min, and the release of [^{3}H]serotonin was measured as described. Control (△) indicates the action of thrombin and collagen in the absence of indomethacin. Results are the average of four experiments ± SEM. Total platelet radioactivity in 100-μl samples was 225,363 ± 2568 (mean ± SEM; n = 4).

DISCUSSION

This study demonstrates that leupeptin—a protease inhibitor—plus indomethacin—a cyclooxygenase inhibitor—synergistically inhibit the effect of thrombin and thrombin/collagen on human platelets.

Leupeptin inhibits all platelet responses elicited by thrombin and trypsin (8), although it does not inhibit thrombin's proteolytic activity or its binding to the platelet surface (9–11). However, leupeptin does not inhibit platelet activation induced by collagen, which is another relevant platelet physiological agonist (8). Indomethacin effectively inhibits collagen-induced platelet activation, but it does not completely inhibit the effect of thrombin on platelets (1). This study demonstrates that a combination of leupeptin and indomethacin effectively inhibits platelet activation induced by both agonists. When both drugs are administered together, full platelet inhibition occurs at doses that are unable to prevent platelet activation when each inhibitor is used alone.

The effects of indomethacin and leupeptin can be explained on the basis of inhibition of platelet cyclooxygenase and proteolysis, respectively. Indomethacin inhibits cyclooxygenase, which catalyzes the formation of endoperoxides and thromboxane A_2. On the other hand, proteases play a role in platelet aggregation and, accordingly, different protease inhibitors and substrates have been tested for their inhibitory activity on platelet aggregation (12). Recently, it has been shown that a platelet Ca^{2+}-dependent protease is activated during aggregation (13), and two specific proteins are hydrolyzed (14, 15). Ca^{2+}-dependent proteolysis has been proposed to play a role in agonist-induced expression of fibrinogen receptor (9), in the irreversible activation of protein kinase C (16), and in the regulation of platelet phospholipases (8). In all of these processes in which Ca^{2+}-dependent proteolysis might be involved, leupeptin is an effective inhibitor (8, 9, 13–15).

In recent years nonsteroidal antiinflammatory drugs such as aspirin and sulfinpyrazone have been used to treat cerebral ischemia and cardiovascular disease (2, 3). There has been a tendency to lower the dose of these drugs (from 1500 to 600 mg daily) (4, 5) in an attempt to reduce side effects. The use of leupeptin and related peptides has never been clinically tested in the treatment of thromboembolic diseases. However, these compounds are currently under investigation for the treatment of muscular dystrophy in children. The synergism between protease and cyclooxygenase inhibitors described here may have important consequences in antiplatelet therapy; it may allow further reduction in the dose of cyclooxygenase inhibitors if given in combination with protease inhibitors. Furthermore, the inhibitory activity of leupeptin on thrombin-induced platelet activation may be useful when nonsteroidal antiinflammatory drugs alone are ineffective antithrombotic agents because they do not inhibit the thrombin-mediated pathway in thrombosis (1).

We thank Lee Rose for her skillful preparation of this manuscript and Allen Jones for editing the manuscript.

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