**Brief Communication**

**Collagen-Stimulated Human Platelet Aggregation Is Mediated by Endogenous Calcium-Activated Neutral Protease**

Teruhiko Toyo-oka, Wee Soo Shin, Yoko Okai, Yoshiyuki Dan, Minoru Morita, Masahiko Iizuka and Tsuneaki Sugimoto

To clarify the physiological role of calcium-activated neutral protease (CANP) in human platelets, we loaded the platelets with a Ca\(^{2+}\)-sensitive fluorescent dye, fura-2, and measured the degree of aggregation, cytosolic calcium ion concentration ([Ca\(^{2+}\)]\(_i\)), and proteolysis by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE). At physiological concentration of Ca\(^{2+}\) (1 mM) in the incubation medium, [Ca\(^{2+}\)]\(_i\) was below 0.5 \(\mu\)M and platelet aggregation was not shown. Ionomycin (0.15 \(\mu\)M) or collagen (50 \(\mu\)g/ml), but not ADP (10 \(\mu\)M), sharply enhanced the [Ca\(^{2+}\)]\(_i\) to near 1 \(\mu\)M and caused the aggregation. A calcium entry blocker, verapamil, completely abolished both the [Ca\(^{2+}\)]\(_i\) rise and the aggregation. NCO-700, a membrane permeable inhibitor against cysteine proteases (including CANP), dose-dependently blocked the aggregation but did not change the [Ca\(^{2+}\)]\(_i\) transient. SDS-PAGE revealed that filamin, talin, and 70 kDa protein were specifically degraded when platelets were aggregated by ionomycin or collagen and that the proteolysis was not observed when the aggregation was blocked by verapamil or NCO-700. These data provided evidence that Ca\(^{2+}\) entry exceeding 0.5 \(\mu\)M is essential, but not sufficient per se, and that activation of cysteine protease, most likely CANP, is involved in the platelet aggregation by collagen or calcium ionophore. (Circulation Research 1989; 64:407-410)

The exact process of platelet aggregation is still obscure. Several mechanisms, including arachidonic acid release/thromboxane A\(_2\) or phosphatidylinositide/protein kinase C systems, have been described. In any case, calcium entry into cytosol precedes the aggregation. Human platelets, as well as other tissues, include a cysteine protease, calcium-activated neutral protease (CANP), though, excepting myofibrillar protein degradation in pathophysiological conditions, physiological function of the enzyme is unclear. Very recently, a cysteine protease inhibitor, NCO-700, was synthesized and found to be inert in living animals, to pass through the cell membrane, and to block proteolysis in living muscle cells.

**Materials and Methods**

**Isolation of Human Platelets and Loading of Fura-2**

Venous blood from healthy human volunteers \((n=6)\) was sampled in the presence of sodium citrate (0.8%) and centrifuged at 800g for 10 minutes. Fura-2-AM (Dojin Chemicals, Tokyo) was added to the supernatant to a final concentration of 1 \(\mu\)M and incubated at 37°C for 30 minutes. The supernatant was centrifuged at 3,000g for 15 minutes, and the pellet was gently suspended in 5 ml of a solution (A) that contained 150 mM NaCl, 20 mM Tris-Cl buffer (pH 7.4), and 100 mg/dl glucose. The final number of platelets was counted by a Coulter counter and adjusted to 5.0x10\(^7\)/\(\mu\)l with solution A.

**Measurement of Platelet Aggregation and Calcium Transient**

An aliquot of prepared platelet suspension was incubated in the absence or presence of NCO-700 (10 \(\mu\)M to 1 mM in final concentration) for 5 minutes.
at 37°C, mixed with Ca²⁺ (1.0 mM), and then stimulated by collagen (50 µg/ml), ionomycin (0.15 µM), or ADP (10 µM). In all steps, platelet aggregation was monitored with a spectrometer.

With the same sample and the same conditions, [Ca²⁺], was assayed with double wavelength excitation (340 and 380 nm) and single emission (502 nm) fluorescent spectrophotometry developed by Tsien's group. At each step, after addition of reagent, excitation wave length was scanned from 300 to 420 nm and recorded on an x-y chart. Control experiment of the same platelets without fura-2 loading was performed, and autofluorescence was subtracted from the total fluorescence curve. For the calculation of free Ca²⁺ concentration, a calibration line was constructed with 5 mM Ca²⁺-ethyleneglycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid buffer and 10 µM free acid fura-2.

Assay of Phospholipase A₂ or C Activity
To examine direct effect of NCO-700 on phospholipase A₂ or phospholipase C, these enzyme activity in isolated human platelets was measured in the absence or presence of NCO-700, following the method of Teramoto et al. or Hofmann and Majerus, respectively. β-[1-¹⁴C]Linoleyl-L-α-phosphatidylcholine and L-α-[³H]-labeled myoinositol were purchased from Amersham, Arlington Heights, Illinois, and New England Nuclear, Boston, Massachusetts, respectively.

Miscellaneous
Before and after the [Ca²⁺], measurement, an aliquot was sampled to check proteolysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized with silver staining. Reagents used were of analytical grade, and water was redistilled before use. NCO-700 was donated by Dr. M. Masaki, Nippon Chemiphar, Tokyo.

Results
Aggregometry of Platelets
In the absence or the presence of the physiological concentration of calcium (1 mM) in the incubation medium, platelet aggregation did not take place (Figures 1 and 2). Collagen (50 µg/ml) and ionomycin (0.15 µM) stimulated the platelet aggregation (Figure 1, C, I). ADP up to 10 µM did not cause platelet aggregation. Preincubation with verapamil (1 mM) completely blocked the aggregation (Figure 1, C+V). Cysteine protease inhibitor NCO-700 also inhibited the platelet aggregation with ionomycin (Figure 1, I+N) or collagen (Figure 2). The aggregation was completely blocked at 1 mM NCO-700 (Figure 2B). Its effect was dose-dependent between 10 µM and 1 mM (Figure 2, C-E), and the concentration necessary to block the aggregation by 50% was about 20 µM.

Intracellular Ca²⁺ Transient in Platelet Aggregation
As summarized in Figure 3, the basal level of [Ca²⁺], before addition of calcium was 80±38 nM (mean±SEM, n=6). Physiological concentration of Ca²⁺ (1 mM) in the incubation medium increased the [Ca²⁺], fourfold to 350±63 nM. Stimulation by collagen (50 µg/ml) sharply enhanced the [Ca²⁺], to above 0.8 µM (Figure 3, unfilled triangles) within 5 minutes. Ionomycin (0.15 µM) raised the [Ca²⁺], even more sharply (unfilled squares). A calcium entry blocker, verapamil (1 mM), did not interfere with the measurement of [Ca²⁺], and blocked the rise of [Ca²⁺], caused by collagen (Figure 3, cross marks), indicating that verapamil surely blocked calcium entry in human platelets. These data demonstrated that the threshold of about 500 nM must be exceeded for platelet aggregation. The cysteine protease inhibitor NCO-700 (1 mM) had no effect on the rise of [Ca²⁺], after the stimulation of collagen (50 µg/ml, filled triangles) or ionomycin (0.15 µM, filled squares). These results indicated that the inhibitory action of NCO-700 did not originate from the calcium entry blocking effect but suggested an involvement beyond the calcium entry step.
FIGURE 3. Intracellular Ca²⁺ transient of fura-2-loaded human platelets in control (C), after addition of 1.0 mM Ca²⁺ (Ca), after stimulation with collagen (50 μg/ml) in the absence (●) or presence of NCO-700 (1 mM, ▲) or after stimulation with ionomycin (0.15 μM) in the absence (○) or presence of NCO-700 (1 mM, ■). All conditions were adjusted to those of the previous aggregometry. Each result denotes the mean±SEM (n=6). Note that a calcium entry blocker, verapamil (0.1 mM), completely inhibited the rise of intracellular Ca²⁺ concentration (X).

SDS-PAGE of Platelet Proteins Before or After Stimulation

After staining by silver nitrate, isolated human platelets showed more than 30 protein bands (Figure 4A). Physiological concentration of Ca²⁺ (1 mM) caused a minor extra band at 180 kDa (Figure 4B).

FIGURE 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the control human platelets (A), after addition of Ca²⁺ (1.0 mM) to the control (B), after stimulation with collagen (50 μg/ml) when the aggregation was blocked with calcium entry blocker, verapamil (1 mM, C), collagen alone which showed platelet aggregation (D), or collagen when the aggregation was blocked with cysteine protease inhibitor, NCO-700 (1 mM, E). Note that protein bands indicated by arrow mark in lane D became less intense only when platelet aggregation took place.

TABLE 1. Relation Among Ca²⁺ Entry Into Cytoplasm, Degradation of Constituent Proteins, and Platelet Aggregation

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Ca²⁺ entry</th>
<th>Proteolysis</th>
<th>Aggregation</th>
</tr>
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<tbody>
<tr>
<td>Collagen</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Collagen+verapamil</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collagen+NCO-700</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ionomycin+NCO-700</td>
<td>+</td>
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The origin of the protein was not pursued because this protein band was not directly correlated with the platelet aggregation.

When the aggregation was blocked by verapamil, no change was documented between the samples before and after the stimulation by collagen (Figure 4C). In contrast, collagen-stimulated platelets revealed a definite density reduction of three protein bands at 250, 235, and 70 kDa (Figure 4D). NCO-700 treatment of platelets completely inhibited the proteolysis, and there was no change in the mobility or density of protein bands compared with the sample before the stimulation (Figure 4E).

Effect of NCO-700 on Activity of Phospholipase A₂ or C

To examine the NCO-700 effect on phospholipase A₂ or C, we measured their activities in the absence and presence of the reagent. NCO-700 revealed no definite effect on phospholipase A₂ activity: 24.3±1.4 nmol/2 hrs without NCO-700 and 21.4±0.5 or 22.6±0.5 nmol/2 hrs at 10⁻⁴ M or 10⁻³ M NCO-700, respectively. The activity of phospholipase C was not inhibited but rather raised by high concentration of NCO-700: 10.2±1.8 nmol/2 hrs without NCO-700 and 15.6±0.5 (p<0.05) or 12.9±1.8 nmol/2 hrs at 10⁻⁴ or 10⁻³ M NCO-700, respectively.

Discussion

Calcium ionophore, ionomycin, or collagen stimulated Ca²⁺ entry, platelet aggregation, and the degradation of 250, 235, and 70 kDa proteins. From the molecular weight and preferential degradation by CANP, we tentatively identified the 250 kDa, 235 kDa, and 70 kDa protein as filamin (actin-binding protein),²⁰ talin,²¹ and glycoprotein Ibb,²² respectively. In contrast, a calcium entry blocker, verapamil, inhibited the [Ca²⁺]j, rise, platelet aggregation, and the hydrolysis of these proteins. These data provided evidence that calcium entry from outside in excess of 0.5 μM was essential for platelet aggregation. However, it was not sufficient per se to cause the aggregation, and cysteine protease activation was mandatory for the aggregation because NCO-700, an inhibitor against cysteine protease (including CANP), blocked both platelet aggregation and the proteolysis in spite of Ca²⁺ entry. Simplified results are summarized in Table 1.

Rink et al have reported that [Ca²⁺]j threshold for aggregation of human platelets was 2 μM,² higher...
than the present data. This discrepancy might be secondary to the high concentration of quin2 that is needed for a detectable difference of [Ca\textsuperscript{2+}]. Both quin2 and fura-2 are Ca\textsuperscript{2+} chelators and attenuate the [Ca\textsuperscript{2+}], transient. To follow an exact time course of the [Ca\textsuperscript{2+}], these fluorescent dye concentrations should be minimized with more-sensitive fura-2. Furthermore, present results confirmed the data of Ware et al that quin2 loaded human platelets did not aggregate with ADF stimulation.\textsuperscript{22}

NCO-700 is a cysteine protease inhibitor like leupeptin or antipain;\textsuperscript{23} it is not specific for CANP but blocks other cysteine proteases such as cathepsin B, H, or L that are included in lysosomes. However, the reagent is more inert to living cells than leupeptin or antipain, as we reported earlier.\textsuperscript{12,13} It would be reasonable to assume that the protease responsible for platelet aggregation is CANP, because CANP requires Ca\textsuperscript{2+} for the activation.\textsuperscript{3-7} Actually, Fox et al demonstrated that CANP isolated from human platelets degraded filamin (250 kDa, actin-binding protein)\textsuperscript{20} and talin (235 kDa protein).\textsuperscript{20,21} These in vitro studies by isolated protease and substrate proteins are in complete agreement with the present results of whole platelets and might provide evidence that CANP among cysteine proteases is activated in platelet aggregation by ionomycin or collagen.

The involvement of the lysosomal proteases cathepsin B, H, or L in platelet aggregation is not likely because these cathepsins are insensitive to Ca\textsuperscript{2+} and require acidic pH for their activation. A more complicated mechanism to explain Ca\textsuperscript{2+}-triggered proteolysis could be the involvement of phospholipase A\textsubscript{2} or C, which is Ca\textsuperscript{2+}-dependent.\textsuperscript{24} However, the possibility was excluded because NCO-700 did not inhibit the activity of these phospholipases. To access the pathway of platelet aggregation, identification of the protein responsible for the platelet aggregation is needed in a more elaborate study.

Acknowledgements

The authors are grateful to Dr. R.Y. Tsien, Department of Neuroscience, University of California, Berkeley, for kind discussion on methodology; Dr. N. Yoshida, Jichi Medical School, Tochigi, for his discussion on results; and Mr. K. Hara for technical assistance in the assay of phospholipases.

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Collagen-stimulated human platelet aggregation is mediated by endogenous calcium-activated neutral protease.

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Circ Res. 1989;64:407-410
doi: 10.1161/01.RES.64.2.407

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