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L-amino acid oxidase from *Naja atra* venom activates and binds to human platelets

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An *L*-amino acid oxidase (LAAO), NA-LAAO, was purified from the venom of *Naja atra*. Its N-terminal sequence shows great similarity with LAAOs from other snake venoms. NA-LAAO dose-dependently induced aggregation of washed human platelets. However, it had no activity on platelets in platelet-rich plasma. A low concentration of NA-LAAO greatly promoted the effect of hydrogen peroxide, whereas hydrogen peroxide itself had little activation effect on platelets. NA-LAAO induced tyrosine phosphorylation of a number of platelet proteins including Src kinase, spleen tyrosine kinase, and phospholipase C γ2. Unlike convulxin, Fc receptor γ chain and T lymphocyte adapter protein are not phosphorylated in NA-LAAO-activated platelets, suggesting an activation mechanism different from the glycoprotein VI pathway. Catalase inhibited the platelet aggregation and platelet protein phosphorylation induced by NA-LAAO. NA-LAAO bound to fixed platelets as well as to platelet lysates of Western blots. Furthermore, affinity chromatography of platelet proteins on an NA-LAAO-Sepharose 4B column isolated a few platelet membrane proteins, suggesting that binding of NA-LAAO to the platelet membrane might play a role in its action on platelets.

Keywords *L*-amino acid oxidase; *Naja atra*; platelet; hydrogen peroxide

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt; biotin-NHS, biotinamidocaproate *N*-hydroxysuccinimide ester; BSA, bovine serum albumin; Fcγ, Fc receptor γ chain; FITC, fluorescein-isothiocyanate; GPVI, glycoprotein VI; LAAO, *L*-amino acid oxidase; LAT, T lymphocyte adapter protein; NA-LAAO, *L*-amino acid oxidase from *Naja atra* venom; NBT, *p*-nitro blue tetrazolium chloride; PBS, phosphate-buffered saline; PLC, phospholipase C; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Src, Src kinase; Syk, spleen tyrosine kinase; TBS, Tris-buffered saline.

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Venom *L*-amino acid oxidases (LAAOs) are homodimeric flavoenzymes that catalyze the oxidative deamination of an *L*-amino acid substrate to an α-keto acid along with the production of ammonia and hydrogen peroxide. They are widely distributed in venomous snake families of Viperidae, Crotalidae and Elapidae [1]. Each subunit contains three domains: an FAD-binding domain; a substrate-binding domain; and a helical domain [2]. Although the mechanisms are uncertain, venom LAAOs are reported to have various biological activities including induction of apoptosis, induction of oedema and haemolysis, antibacterial function, and platelet activation or inhibition. All the effects are thought to be at least partly related to H_2O_2 production because catalase, an H_2O_2 scavenger, inhibits the actions of venom LAAOs [1].

The reported effects of LAAOs on platelets are quite controversial. LAAO from *Echis colorata* inhibits ADP-induced platelet aggregation. LAAOs from *Agkistrodon halys blomhoffii*, *Naja naja kaouthia*, and king cobra inhibit agonist-induced or shear stress-induced platelet aggregation [3–5]. These reports suggested that the interaction between activated platelet integrin $\alpha_{IIb}\beta_3$ and fibrinogen was inhibited by the continuous generation of H_2O_2. LAAOs from some other snakes have been reported to have the totally opposite effect on platelets. LAAOs from *Eristocophis macmahoni*, *Bothrops alternatus*, and *Trimeresurus jerdonii* induce human platelet aggregation through formation of H_2O_2 [6–8]. It is still not clear how H_2O_2 functions in LAAO-induced platelet aggregation.

It is also possible that LAAOs activate platelets in a receptor-dependent way. Several recent studies showed that H_2O_2 production might not be the whole story for the biological activities of LAAOs. LAAO from *A. halys* showed many binding and cytotoxic effects on different cell lines [9]. Hydrogen peroxide generated in the enzymatic reactions was not sufficient to explain the degree to which bacterial growth was inhibited by a *D*-amino acid oxidase from hog kidney and an LAAO from the venom of *A.*

halys. A fluorescence labeling assay showed that both of these enzymes bind to the surface of bacteria [10], and a novel LAAO from *Trimeresurus stejnegeri* showed dose-dependent inhibition on HIV-1 infection and replication. The presence of catalase resulted in an increase in its antiviral selectivity. However, under the same conditions, no anti-HIV-1 activity was observed by exogenous addition of H_2O_2 [11].

Here, we report the purification and characterization of an LAAO from *Naja atra* venom, named NA-LAAO. We show that it activates washed human platelets but not platelets in platelet-rich plasma, and binds directly to platelets.

Materials and Methods

Materials

Lyophilized *N. atra* venom was from Yunnan Province, China. BSA, EDTA, protein A-Sepharose, peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies, fura-2/AM, FITC, BCIP, streptavidin-alkaline phosphatase, biotin-NHS, NBT, tetramethyl benzidine, horseradish peroxidase, catalase, *L*-leucine, and Triton X-100 were from Sigma (St. Louis, USA). Hydrogen peroxide was from Merck (Darmstadt, Germany). Sepharose 4B was from Amersham Biosciences (Piscataway, USA). The SuperSignal chemiluminescence detection system was from Pierce (Rockford, USA), and autoradiography (Fuji RX) films were from FujiFilm (Dielsdorf, Switzerland). Antiphosphotyrosine monoclonal antibody 4G10 was from Lucernachem (Luzern, Switzerland). PVDF membranes (PolyScreen) were from Dupont NEN (Boston, USA). Anti-LAT, anti-PLCγ2, anti-Src, and anti-Syk antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). EMD 132338, an $\alpha_{IIb}\beta_3$ inhibitor, was a kind gift from Merck. Convulxin was purified as described previously [12].

Purification of LAAO

Chromatography of *N. atra* venom on an SP-Sephadex C-25 column (5 cm×60 cm; Pharmacia, Uppsala, Sweden) was carried out as described previously [13]. Briefly, crude venom of *N. atra* (5 g) was dissolved in 20 ml of 50 mM sodium acetate buffer (pH 5.8) and applied to the SP-Sephadex C-25 column pre-equilibrated with the same buffer. Unbound protein was washed out with the same buffer and then a gradient of 0–1 M NaCl in the same buffer was applied at a flow rate of 60 ml/h. Fractions containing LAAO were collected, lyophilised, and loaded on a Fractogel EMD BioSEC 650(S) gel filtration column (1.6 cm×120 cm; Merck, Whitehouse Station, USA) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl, and then eluted at 30 ml/h for 15 h collecting 5 ml fractions. Active fractions were pooled and dialysed against 20 mM Tris-HCl buffer (pH 7.4) and applied to a Bio-Scale Q2 column (Bio-Rad, Hercules, USA) equilibrated with the same buffer. The bound proteins were eluted with a 0–0.6 M NaCl gradient in the same buffer at a flow rate of 30 ml/h. The active fractions were analyzed by SDS-PAGE silver staining and stored at 4 ºC. The N-terminal amino acid sequence was determined by ABI model 476A protein sequencer (Applied Biosystems, Foster City, USA).

LAAO activity assay

A reaction mixture (200 μl) containing 1 mM *L*-leucine, 10 μM tetramethyl benzidine, and 10 mU/ml horseradish peroxidase in 0.1 M Tris-HCl buffer (pH 8.5) was incubated at 25 ºC. The reaction was started by adding crude fractions or purified NA-LAAO and monitored at 450 nm over 10 min. One unit of the enzyme was defined as the oxidation of 1 μmol of *L*-leucine per minute.

SDS-PAGE, silver staining, and protein determination

SDS-PAGE was carried out according to Laemmli [14] with a 7%–17% acrylamide gradient, and the gel was silver stained by the method of Morrissey [15]. Protein determination was carried out by bicinchoninic acid protein assay (Pierce) with bovine albumin as standard.

Preparation of washed platelets and platelet aggregation

Human platelets were isolated from human blood obtained from the Central Laboratory of the Swiss Red Cross Blood Transfusion Service (Berne, Switzerland). For 100 ml of human blood, 30 ml of 100 mM citrate, pH 6.5, was added. Platelet-rich plasma and the platelet pellet were isolated by successive centrifugation. Platelets were resuspended with buffer B containing 113 mM NaCl, 4.3 mM K_2HPO_4, 24.4 mM NaH_2PO_4, and 5.5 mM glucose, pH 6.5, and centrifuged at 250 *g* for 5 min. The platelet-rich supernatant was centrifuged at 1000 *g* for 10 min, and the platelets were washed once more with buffer B. Washed platelets were resuspended in buffer C containing 20 mM HEPES, 140 mM NaCl, 4 mM KCl, and 5.5 mM glucose, pH 7.4, and the platelet count was adjusted to 5×10^8 platelets/ml by dilution with buffer C. Samples were kept at room temperature until used for aggregation studies. Before aggregation analysis, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ were added and the platelets were incubated at 37 ºC for 2 min. Platelet aggregation was measured by light transmission

in an aggregometer (LumiTec, Paris, France) with continuous stirring at 1100 rpm at 37 ºC.

Time course of tyrosine phosphorylation in platelets

Platelets were treated for aggregation. Aliquots (45 μl) were taken at fixed time points and the platelet suspension was lysed by adding the aliquots into 5 μl HEPES containing 10% SDS, 10 mM *N*-ethylmaleimide, 20 mM sodium orthovanadate, and 20 mM EDTA. After centrifugation, the supernatants were analyzed by a 7%–17% gradient SDS-polyacrylamide gel and electroblotted onto a PVDF membrane. The membrane was incubated with 2% BSA in TBS overnight. Tyrosine phosphorylated proteins were detected by 4G10 monoclonal antibody followed by peroxidase-coupled rabbit anti-mouse secondary antibodies. Bound antibodies were detected using chemiluminescence.

Platelet biotinylation and NA-LAAO-Sepharose 4B affinity chromatography

Human platelets were isolated from buffy coats as described above but in the presence of 10 μM iloprost. Washed platelets were diluted with PBS to 5×10^9 platelets/ml and incubated with 10 μg biotin-NHS for 1 h at room temperature. Free biotin-NHS was removed by washing the platelets three times with PBS, pH 6.8. Biotinylated platelets were solubilized in PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, 2 mM *N*-ethylmaleimide, and 2 mM sodium orthovanadate. After centrifugation (12,000 *g* for 30 min at 4 ºC), the supernatant was applied to a column of NA-LAAO-Sepharose 4B and eluted successively with TBS containing 0.2% octanoyl-*N*-methylglucamide and 0.1% or 0.5% SDS. The eluted fractions were analyzed using SDS-PAGE silver stain and Western blotting detected with phosphatase-labeled streptavidin followed by NBT/BCIP.

Binding of biotin-labeled NA-LAAO to blots of platelet lysates

NA-LAAO in 50 mM $NaHCO_3$ was mixed with biotin-NHS dissolved in Me_2SO (0.25 mg biotin-NHS/mg protein). The mixture was incubated at room temperature for 2 h. Unlabeled biotin-NHS was removed by loading the sample to a Sephadex G-25 column eluted with TBS. Washed platelets (5×10^8 platelets/ml) were lysed in HEPES containing 1% Triton X-100, 1 mM *N*-ethylmaleimide, 2 mM sodium orthovanadate, and 2 mM EDTA. After centrifugation, the supernatants were separated on a 7%–17% gradient SDS-PAGE and transferred onto a PVDF membrane. After blocking with 2% BSA overnight, a solution containing biotin-labeled NA-LAAO was added to the membrane and incubated for 1 h. The membrane was washed four times with TBS containing 0.1% Tween 20. Bound biotin-NA-LAAO was detected with phosphatase-conjugated streptavidin followed by NBT/BCIP.

Immunoprecipitation

Aliquots (500 μl) of resting as well as activated platelets (5×10^8 platelets/ml) were solubilized in Tris-buffered saline containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM *N*-ethylmaleimide, 2 mM benzamidine, and 2 mM sodium orthovanadate. After centrifugation, platelet lysates, precleared with protein A-Sepharose, were stirred for 2 h with specific antibodies before adding 20 μl protein A-Sepharose followed by 6–8 h incubation. After washing, proteins were eluted from the protein A-Sepharose by boiling with 40 μl of 100 mM Tris-HCl, pH 7.5, 5% SDS, and 5 mM EDTA.

Flow cytometry analysis of FITC-NA-LAAO binding to fixed platelets

Washed platelets were fixed with 1% formaldehyde in TBS at room temperature for 0.5 h. The fixed platelets were washed twice with TBS and then resuspended in TBS at 5×10^7 platelets/ml. Then FITC-NA-LAAO was added to 0.1 ml platelets and shaken for 10 min at room temperature in the dark. The platelets were washed twice with TBS and then analyzed by flow cytometry. For competent assay, 10-fold excess of unlabeled NA-LAAO was incubated with the platelets for 10 min at room temperature before adding FITC-labeled NA-LAAO.

Results

Purification of NA-LAAO from *N. atra* venom

NA-LAAO was purified from *N. atra* venom by a three-step chromatography protocol including cation ion exchange on an SP-Sephadex C-25 column, gel filtration on a Fractogel EMD BioSEC 650(S) column [**Fig. 1(A)**], and anion ion exchange on a Bio-Rad Q2 column [**Fig. 1 (B)**]. The purified NA-LAAO was homogeneous on SDS-PAGE detected by silver staining, which showed it was a pure protein [**Fig. 1(B)**, insert]. The N-terminal sequence of NA-LAAO was determined to be DDRRSPLEEC, which has high similarity to other venom LAAOs and is identical to the LAAO from *N. kaouthia* venom [**Fig. 1(C)**] [4]. The enzyme activity of NA-LAAO was 38.4 U/(mg·min).

NA-LAAO activated platelets

A high dose (15 μg/ml) of NA-LAAO directly activated washed platelets (**Fig. 2**). However, it had no activation

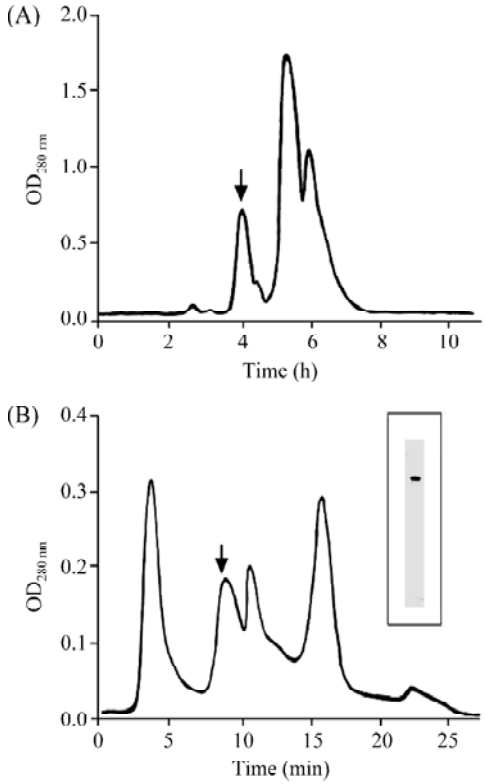

Fig. 1 Purification of an *L*-amino acid oxidase (LAAO) from the venom of *Naja atra* (NA-LAAO) (A) Separation of LAAO activity containing fractions from a SP-Sephadex C-25 column by a Fractogel EMD BioSEC 650(S) gel filtration column (1.6 cm×120 cm). (B) Further purification of NA-LAAO on a Bio-Scale Q2 column. The arrows indicate the fractions containing NA-LAAO. Insert, NA-LAAO protein. (C) Alignment of N-terminal sequence of venom LAAOs. Amino acid residues identical to the first line are shaded. OD, optical density.

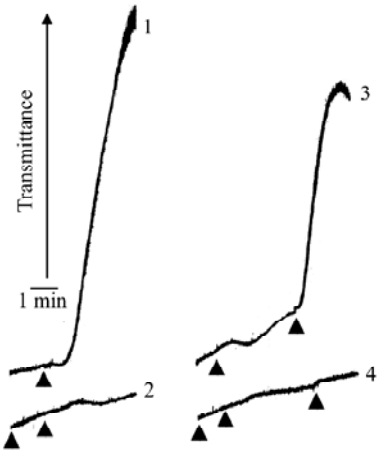

Fig. 2 Human platelet aggregation induced by high concentration of an amino acid oxidase from the venom of *Naja atra* (NA-LAAO) and low concentration of NA-LAAO plus H_2O_2 Washed platelets (5×10^8 platelets/ml, 500 μl) were treated with 15 μg/ml NA-LAAO (line 1), or incubated with 600 U/ml catalase before adding 15 μg/ml NA-LAAO (line 2). Washed platelets were incubated with 1.5 μg/ml NA-LAAO for 3 min then H_2O_2 (40 μM) was added (line 3), or incubated with 600 U/ml of catalase before adding the NA-LAAO and H_2O_2 (line 4).

effect on platelets in platelet-rich plasma (data not shown). Catalase (600 U/ml) inhibited the platelet aggregation (**Fig. 2**). Several platelet proteins were phosphorylated in NA-LAAO-induced platelet aggregation [**Fig. 3(A,B)**]. Some of them were identified by immunoprecipitation to be Src, PLCγ2, and Syk. However, unlike in convulxin-activated platelets [11], Fcγ and LAT were not phosphorylated in NA-LAAO-activated platelets [**Fig. 3(C)**]. The phosphorylation of the proteins was also inhibited by catalase. In contrast, a low dose of NA-LAAO (1.5 μg/ml) did not induce platelet aggregation, but it potentiated the action of H_2O_2 (40 μM). H_2O_2 itself did not induce platelet aggregation and had little effect on platelet protein phosphorylation. Incubation of low doses of NA-LAAO or H_2O_2 did not change the platelet protein phosphorylation profile. Adding H_2O_2 (40 μM) after incubation of a low dose of NA-LAAO for 3 min induced rapid platelet aggregation and phosphorylation of platelet proteins (**Fig. 3**). The activations were inhibited by catalase (**Figs. 2** and **3**). It is interesting that after frequent freezing and thawing, NA-LAAO retained its enzymatic activity. However, its activity on platelet aggregation was greatly impaired (data not shown).

Full activation of platelets by NA-LAAO requires Ca^{2+} and α_{IIb}/β_3 activation

As shown in **Fig. 4(A)**, EDTA and EMD 132338 inhibited the platelet aggregation induced by NA-LAAO. The inhibitory effect of EDTA was stronger than that of EMD 132338. Also the two inhibitors greatly inhibited the phosphorylation of platelet proteins [**Fig. 4(B)**].

NA-LAAO binds to platelets

NA-LAAO was labeled with FITC and incubated with fixed

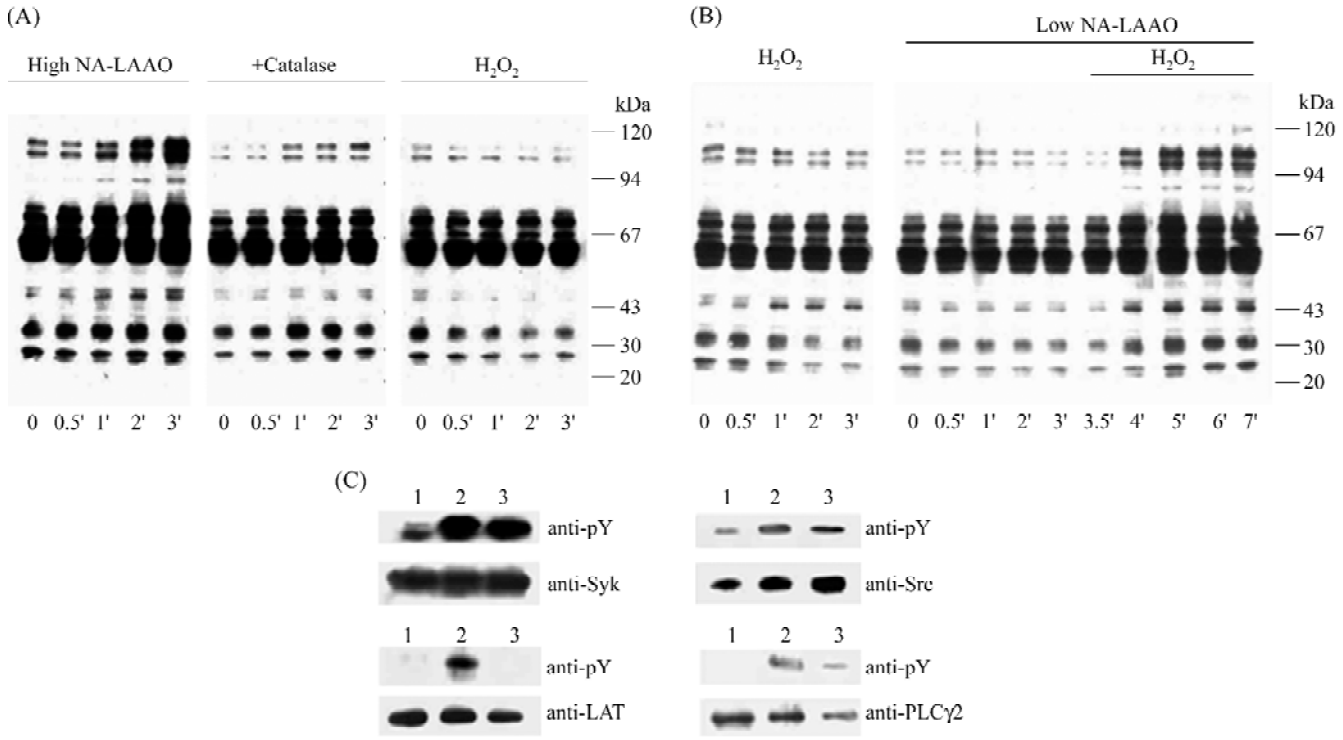

Fig. 3 Time dependence of tyrosine phosphorylation in human platelets activated by high concentration of an amino acid oxidase from the venom of *Naja atra* (NA-LAAO) or low concentration of NA-LAAO plus H_2O_2 (A) Washed platelets were treated with 15 μg/ml NA-LAAO, with 600 U/ml of catalase before adding 15 μg/ml NA-LAAO, or with 40 μM H_2O_2. (B) Washed platelets were treated with 40 μM H_2O_2 or 1.5 μg/ml NA-LAAO and 40 μM H_2O_2. Aliquots (45 μl) were taken at fixed times and the platelet suspension was lysed in sodium dodecyl sulfate (SDS) lysis buffer containing inhibitors. After centrifugation, the supernatant was run on a 7%–17% gradient SDS-polyacrylamide gel then electroblotted onto a polyvinylidene difluoride membrane. The monoclonal antibody 4G10 was used to detect tyrosine phosphorylated protein followed by peroxidase-coupled rabbit anti-mouse secondary antibodies. Bound antibody was detected using chemiluminescence. (C) Resting platelets (lane 1) or platelets activated by convulxin (lane 2) or NA-LAAO (lane 3), were lysed by 1.2% Triton X-100 and aliquots of 0.7 ml platelets were used for immunoprecipitation by anti-Src kinase (anti-Src), anti-spleen tyrosine kinase (anti-Syk), anti-T lymphocyte adapter protein (anti-LAT), or anti-phospholipase Cγ2 (anti-PLCγ2) antibodies. After SDS-polyacrylamide gel electrophoresis and Western blot analysis, the membrane was treated with 4G10 anti-phosphotyrosine antibody followed by peroxidase-coupled rabbit anti-mouse secondary antibodies and chemiluminescence. The membrane was stripped and stained with anti-Src, anti-Syk, anti-LAT or anti-PLCγ2 antibodies.

platelets. In flow cytometry assay, FITC-NA-LAAO bound to platelets and the binding was inhibited by 10-fold excess unlabeled NA-LAAO [**Fig. 5(A)**]. Furthermore, biotin-labeled NA-LAAO could bind to platelet lysate immobilized on a PVDF membrane. A protein band of 66 kDa and a 72 kDa doublet were seen on the membrane incubated with biotin-NA-LAAO and stained with avidin-coagulated alkaline phosphatase followed by NBT/BCIP [**Fig. 5(B)**].

Platelet proteins bind to NA-LAAO

Biotin-labeled platelets lysed by Triton X-100 were loaded onto an NA-LAAO-Sepharose 4B column. The fractions were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with avidin-coupled alkaline phosphatase and stained with NBT/BCIP (**Fig. 6**) or with rabbit anti-GPIb or rabbit anti-GPVI antibodies (data not shown). There were several protein bands on the membrane stained with avidin-coupled phosphatase and NBT/BCIP under both reduced and non-reduced conditions. These proteins were neither GPVI nor GPIb, as checked by Western blot analysis.

Discussion

This study reports the isolation and characterization of NA-LAAO, an LAAO from the venom of *N. atra*. Its N-terminal sequence is highly similar to those of other known venom LAAOs. Because the reported functions of venom LAAOs on platelets are controversial, we investigated its activities on human platelets.

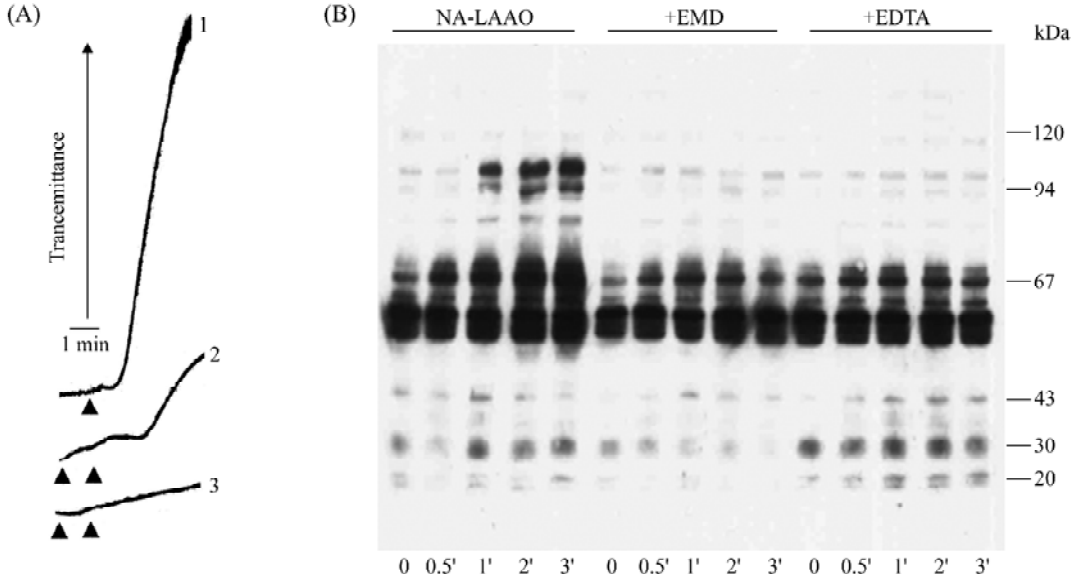

Fig. 4 Inhibitory effect of EDTA and EMD 132338, an $\alpha_{IIb}\beta_3$ inhibitor, on human platelet aggregation and signaling induced by an amino acid oxidase from the venom of *Naja atr*a (NA-LAAO) (A) Washed platelets (5×10^8 platelets/ml, 500 μl) were activated by 15 μg NA-LAAO (line 1), or incubated with 5 μM EMD 132238 (line 2) or 5 mM EDTA (line 3) before adding NA-LAAO. (B) Inhibition of NA-LAAO-induced platelet protein tyrosine phosphorylation by EMD 132338 (5 μM) or EDTA (5 mM).

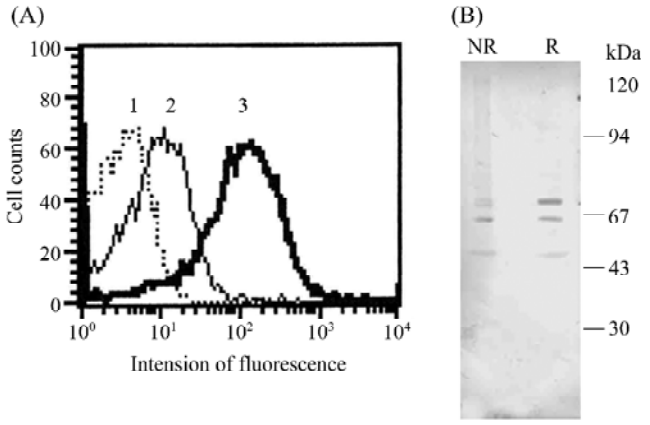

Fig. 5 Amino acid oxidase from the venom of *Naja atr*a (NA-LAAO) binds to fixed human platelets and platelet proteins on a polyvinylidene difluoride (PVDF) membrane (A) Flow cytometric analysis of fluorescein-isothiocyanate (FITC)-labeled NA-LAAO binding to platelets. Fixed platelet control (1); FITC-labeled NA-LAAO binds to fixed platelets (3) and the binding is inhibited by unlabeled NA-LAAO (2). (B) Biotin-labeled NA-LAAO binds to platelet proteins. Triton lysates of platelets were separated on a 7%–17% gradient sodium dodecyl sulfate-polyacrylamide gel and transferred onto a PVDF membrane. After blocking with 2% bovine serum albumin overnight, a solution containing biotin-labeled NA-LAAO was added to the membrane and incubated for 1 h. After washing, bound biotin-NA-LAAO was detected with phosphatase-conjugated streptavidin followed by *p*-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt. NR, non-reduced conditions; R, reduced conditions.

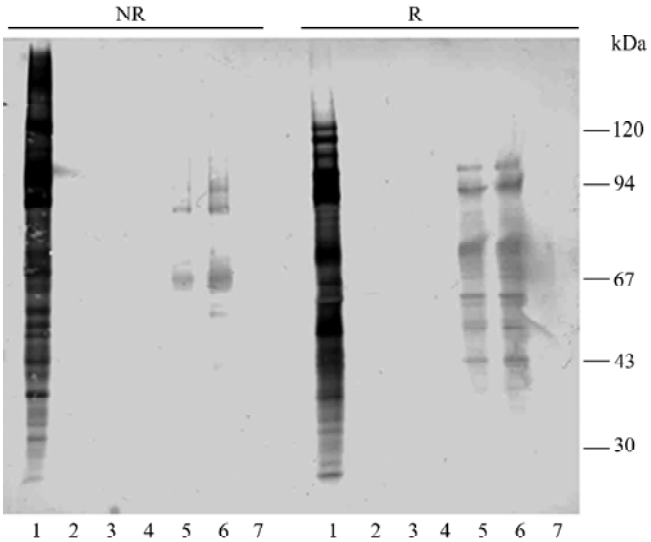

Fig. 6 Binding of biotinylated human platelet proteins to an amino acid oxidase from the venom of *Naja atr*a (NA-LAAO) Biotinylated platelet proteins eluted from NA-LAAO affinity column with increasing amounts of sodium dodecyl sulfate (SDS) were separated by SDS-polyacrylamide gel electrophoresis (7%–17% acrylamide gradient) and the blots were detected with phosphatase-labeled streptavidin followed by *p*-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt. 1, whole platelet lysate; 2–4, the fractions eluted with 0.2% octanoyl-*N*-methylglucamide; 5 and 6, the fractions eluted with 0.1% SDS; 7, the fractions eluted with 0.5% SDS. NR, non-reduced conditions; R, reduced conditions.

The activation or inhibition functions of venom LAAOs on platelets are largely ascribed to their ability to produce H_2O_2 because catalase, an H_2O_2 scavenger, inhibits these effects. However, H_2O_2 production alone is insufficient to account for the effects. There are few reports that H_2O_2 directly induces platelet aggregation. In our experiments, H_2O_2 (2–500 μM) did not induce platelet aggregation (data not shown). However, several lines of evidence showed that the biological actions of LAAOs are only partly dependent on H_2O_2 production, suggesting that there are specific targets or receptors on cells [8–10]. NA-LAAO induced aggregation of washed human platelets at high doses (15 μg/ml). Catalase inhibited this platelet aggregation. However, the amount of H_2O_2 produced by the enzyme is not enough to explain the aggregation as discussed above. Furthermore, a low dose of NA-LAAO (1.5 μg/ml) did not induce platelet aggregation even after long incubation (data not shown). It is likely that the function of NA-LAAO is concentration-dependent. After frequently freezing and thawing, NA-LAAO retained its enzymatic activity as assayed by H_2O_2 production. However, its activity in inducing platelet aggregation was greatly reduced (data not shown). It is possible that freezing and thawing processes affect part of the structure needed to interact with platelets.

Several studies have shown that H_2O_2, a reactive oxygen species, is an intracellular messenger involved in a large number of signal transduction mechanisms, especially those mediated by tyrosine kinases [16–18]. Reactive oxygen species stimulate tyrosine phosphorylation by activating several kinases, such as members of the mitogen-activated protein kinase pathway, Janus kinase, and members of the Src family [19,20]. However, the mechanism remains unknown. A large number of physiological agonists stimulate H_2O_2 production in several cell types, including human platelets [21,22]. For example, collagen-induced platelet aggregation is associated with a burst of H_2O_2 that acts as a second messenger by stimulating arachidonic acid metabolism and the phospholipase C pathway [22]. H_2O_2 added after incubation a low dose of NA-LAAO with platelets induced platelet aggregation. In addition, this treatment induced phosphorylation of a number of platelet proteins including Src, Syk, and PLCγ2, resembling that produced by a high dose of NA-LAAO. As H_2O_2 alone has few effects in activating platelets, it is possible that incubation of NA-LAAO with platelets sensitizes them to H_2O_2 and thus induces platelet aggregation when H_2O_2 is added. $\alpha_{IIb}\beta_3$ inhibition by EMD 132338 greatly reduced the platelet aggregation and phosphorylation of platelet proteins, suggesting that it is activated in NA-LAAO-treated platelets. EDTA, a divalent cation chelator, completely inhibited platelet aggregation induced by NA-LAAO, indicating that calcium influx might play an important role in the activation by NA-LAAO.

Recent studies suggested that H_2O_2 production alone could not explain the biological activities of LAAOs. LAAO from *A. halys* strongly associated with 1210 cells but not HeLa cells and the cytotoxic effect is variable depending on cell lines [9]. In addition, H_2O_2 generated in the enzymatic reactions was not sufficient to explain the degree to which bacterial growth was inhibited by a *D*-amino acid oxidase from hog kidney and an LAAO from the venom of *A. halys*. A fluorescence-labeling assay showed that both of these enzymes could bind to the surface of bacteria [10]. Furthermore, a novel LAAO from *T. stejnegeri* dose-dependently inhibited HIV-1 infection and replication. The presence of catalase resulted in an increase in its antiviral selectivity. However, under the same conditions, no anti-HIV-1 activity was observed by exogenous addition of H_2O_2 [11]. Our results support the above observations. First, FITC-labeled NA-LAAO binds to fixed platelets. This binding is specific because non-labeled enzyme competes for binding with the labeled one. Second, biotin-labeled NA-LAAO binds to platelet lysate immobilized on PVDF membranes. This interaction is also specific and strong because the non-labeled enzyme inhibited the binding and the enzyme recognized the proteins under the stringent conditions of Western blot analysis. Finally, affinity isolation by an NA-LAAO Sepharose 4B column revealed several platelet membrane proteins, although these proteins were not identified in this study.

In conclusion, NA-LAAO from *N. atra* venom induces platelet aggregation. Both H_2O_2 production and binding to platelet membrane proteins might be involved in its action. The enzyme binds to the platelet membrane to enhance the sensitivity of platelets to H_2O_2. At the same time, H_2O_2 released by the enzyme activated platelets by an unknown mechanism.

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