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Regular Article

Allicin and disulfiram enhance platelet integrin αIIbβ3-fibrinogen binding

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ABSTRACT

Introduction: Activation of the platelet receptor αIIbβ3 (glycoprotein IIbIIIa) involves a change in the disulfide bonds pattern in the extra-cellular domain of the receptor. The disulfide-bond reducing agent, dithiothreitol (DTT), can increase integrin activity, and point mutations of specific cysteine residues of the integrin can cause its lockage at the high affinity state. The present study is aimed to support the hypothesis that prevention of specific αIIbβ3 intra-molecular disulfide bond formation increases receptor-ligand binding activity.
Methods: Platelet aggregation was induced by collagen or ADP and epinephrine. Integrin αIIbβ3-fibrinogen binding was evaluated on prostaglandins E_1 (PGE_1)-treated washed platelets or baby hamster kidney (BHK) cells expressing human αIIbβ3. Integrin was directly activated by an anti-ligand induced binding site (LIBS) PT25-2 antibody. The effect of sulfhydryl-reactive agents, such as allicin, glutathione, dithiobis nitrobenzoic acid (DTNB) and disulfiram, was tested on αIIbβ3 activity.
Results: Allicin (40 μM) completely inhibited washed platelets agonist-induced aggregation. Both allicin and disulfiram (40 μM) inhibited αIIbβ3-fibrinogen binding and P-selectin expression in washed platelets. However, there was an increase in αIIbβ3-fibrinogen binding but not P-selectin expression in PGE_1-treated washed platelets activated by PT25-2 antibody. At a high concentration (400 μM) both inhibited αIIbβ3-fibrinogen binding. Similarly, in BHK cells expressing αIIbβ3 activated by PT25-2 antibody, allicin at a low concentration increased αIIbβ3 activity.
Conclusions: Allicin and disulfiram inhibit agonist-induced washed platelet activation probably via inhibition of platelet signaling, but enhance PT25-2 antibody-induced αIIbβ3 integrin activity most likely by preventing reformation of disulfide bridges thereby stabilizing the active conformation of the integrin.

The platelet fibrinogen receptor, integrin αIIbβ3 (glycoprotein IIbIIIa), constitutes an ideal model to study the role of integrin in cell contact interactions. This receptor has the capacity to shift through activation states and can change from a low-affinity state, unable to bind macromolecular ligands, to a ligand-competent state on activated platelets [1]. The process of affinity modulation, mediated by cell signaling ("inside-out" signaling), is an essential part of the integrin function. Furthermore, ligand binding to αIIbβ3 itself modifies the activation state of this integrin, exposing neo-epitopes known as ligand-induced binding sites (LIBS), and leading to post-receptor occupancy events, such as clustering of αIIbβ3 complexes, generation of secondary signals, and cytoskeletal rearrangement, which all contribute to maximal platelet aggregation [1]. However, anti-LIBS antibodies binding to αIIbβ3 result in integrin activation without inside-out signaling [2].

Allicin (diallylthiosulfinate), a chemically unstable and highly reactive molecule, is responsible for the pungent smell of garlic. Fresh garlic clove contains 4-5 mg of allicin and it represents about 70% of the overall thiosulfinates that form when garlic cloves are crushed [3]. Consumption of one-to-three cloves per day results in allicin blood concentration of 6 to 18 μM. Allicin the most studied, biologically active compound of garlic, easily penetrates cellular membranes [4], and possesses a variety of biological effects, including antimicrobial, hypolipidemic, anticancer, and antithrombotic [5–9]. Most of these effects can be attributed to its highly active disulfide bond [-S(O)-S-]. Thus, allicin can react with different sulfhydryl (-SH)-containing proteins (but not disulfide bonds) blocking their free sulfhydryl residues, which prevent them from forming disulfide bonds [10].

Both αIIb and β3 integrin subunits contain highly conserved cysteine residues that form disulfide bonds. It has been suggested that some exist as free sulfhydryls and are directly involved in integrin activation [11]. The reducing agent dithiothreitol (DTT) activates and causes slow progressive platelet aggregation [12].

Others suggest that extra-cellular free thiols and enzymatically catalyzed disulfide bond exchanges are required for acquisition of a

Abbreviations: ADP, adenosine diphosphate; LIBS, ligand induced binding site; BHK, baby hamster kidney; DTT, dithiothreitol; PGE_1, prostaglandin E_1; BSA, bovine serum albumin; DTNB, dithiobis nitrobenzoic acid; GSH, glutathione; PBS, phosphate buffered saline; DMEM, Dulbecco's modified eagle's medium; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

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ligand binding conformation of αIIbβ3 and for sustained integrin ligation [13,14]. The enzyme-mediated disulfide exchange may transiently form free sulfhydryls in the activated integrin to stabilize the integrin-ligand binding.

Redox modulation by DTT and several point mutations in cysteine residues of the b_3 subunit on the EGF domains can lock the αIIbβ3 integrin in its high affinity state [15,16]. This suggests that prevention of specific αIIbβ3 intra-molecular disulfide bond formation increases receptor-ligand binding. To further establish this hypothesis, the effect of allicin, a highly potent sulfhydryl blocker derived from garlic and disulfiram, was tested on αIIbβ3 binding.

Materials and methods

Materials

Prostaglandin E_1 (PGE_1), citric acid, bovine serum albumin (BSA), human serum albumin (essentially fatty acid free, 99% pure) and the thiol blocker reagents dithiobis nitrobenzoic acid (DTNB), glutathione (GSH), fibrinogen, and disulfiram were purchased from Sigma (St. Louis, MO, USA). Collagen, ADP and epinephrine were obtained from DiaMed (Cressier, Switzerland). Mouse monoclonal anti-human αIIbβ3 ligand inducing binding site (LIBS) antibody PT25-2, an αIIbβ3 direct-activator, was purchased from Takara Otsu (Shiga, Japan). Mouse monoclonal anti-human P-selectin antibody, RPE conjugated, was purchased from BioLegend (San Diego, CA, USA). PAC-1, a fibrinogen-mimetic anti-human αIIbβ3 monoclonal antibody, was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Pure allicin was produced by reacting synthetic allicin with immobilized allinase as previously described [17] and generously provided by D. Mirelman (The Weizmann Institute of Science, Rehovot, Israel). Phosphate-buffered saline (PBS), Trypsin B solution, fetal calf serum, glutamine and Dulbecco's modified eagle's medium (DMEM) were purchased from Biological Industries (Beit Haemek, Israel). Lipofectamine reagent and G418 were from Invitrogen (Carlsbad, CA, USA). Hygromycin was from Roche Applied Science (Basel, Switzerland).

Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP)

Blood samples were obtained from healthy volunteers following the Local Institutional Review Board approved protocols. Blood was collected routinely into 3.2% sodium citrate (9:1, blood: anticoagulant ratio). PRP and PPP were prepared by differential centrifugation at 140 g/1880 g for 10 min at room temperature, respectively.

Preparation of washed platelets

Washed platelets were prepared by diluting PRP with PBS (1:3) and incubating the solution for 10 min with PGE_1 (100 ng/ml) and citric acid (5 mM), followed by a 6 min 840 g centrifugation. Supernatant was cleared and platelets were re-suspended in Tyrodes/HEPES buffer (pH 7.4) up to its previous volume.

Platelet aggregation

Washed platelets (225 μl) were incubated for 10 min with allicin. Autologous PPP (25 μl) was added followed by an aggregometer test (Helena Laboratory; PACKS-4, Beaumont, TX, USA) using collagen (5 μg/ml) or ADP (11 μM) and epinephrine (55 μM).

Flow cytometry

Washed platelets diluted 1:10 with PBS were incubated for 30 min (in the dark at room temperature) with ADP (11 μM) and epinephrine (55 μM), or the anti-LIBS αIIbβ3 direct-activator antibody PT25-2 (1:60), together with anti P-selectin and PAC-1, or anti-fibrinogen antibodies. To determine fibrinogen binding, washed platelet samples were supplied with fibrinogen (0.25 mg/ml). The different thiol-blocker reagents, such as allicin, disulfiram, DTNB and GSH, were also added to the platelets. Samples were diluted with 0.7 ml of cold PBS and analyzed in the EPICS XL Coulter Flow Cytometer (Coulter, Miami, FL, USA).

Testing direct effect of sulfhydryl-blocker reagents on αIIbβ3 integrin activation state

Washed platelets were incubated with PGE_1 (100 ng/ml) for 10 min at room temperature to block their signal transduction, and thus their inside-out αIIbβ3 activation mechanism. Integrin αIIbβ3 on platelets was then directly activated with PT25-2-antibody, while allicin or other sulfhydryl-blocker reagents were added to the solution. The state of specific αIIbβ3 activity was evaluated by measuring PAC-1 binding or fibrinogen binding (using anti-fibrinogen antibodies), while total platelet activation was monitored by measuring P-selectin expression.

Binding assay of BHK cells expressing αIIbβ3

Baby hamster kidney (BHK) cells were transfected with normal αIIb and normal β3 as previously described [18]. Briefly, cells were co-transfected with 1 μg of pCNA3/b3 plasmid and 1 μg of pCEP4/αIIb plasmid, using the reagent lipofectamine, and were selected in a medium containing 0.5 mg/ml hygromycin and 0.7 mg/ml G418. Control cells (non-expressing αIIbβ3) were co-transfected with 1 μg pCNA3 and 1 μg pCEP4, with the same selecting medium as described above. Cells were grown in DMEM medium containing 5% fetal calf serum and 2 mg/ml L-glutamine at 37 °C in the presence of 5% CO_2. Cells were collected from wells by Trypsin-B solution containing 0.25% Trypsin and EDTA diluted 1:2000 in PBS, and incubated for 30 min in a calcium-containing medium and then subjected to binding assays.

Statistical analysis

Data were expressed as mean ± standard deviation. Student's t-test was used to analyze the difference between experimental and control groups and $p<0.05$ was considered as a significant difference.

Results

Effect of allicin on platelet aggregation

The effect of allicin on platelet aggregation was studied by pretreatment of PRP and washed platelets with or without allicin at the indicated concentrations for 10 min (Fig. 1). After incubation, platelet aggregation was induced by collagen or ADP and epinephrine and measured with an aggregometer device.

Platelet aggregation in PRP samples was not inhibited by allicin up to a concentration of 40 μM (Fig. 1A). However, in washed platelet samples aggregation was inhibited by allicin (Fig. 1B). Partial inhibition was observed at allicin concentrations of 2.5 and 10 μM, and nearly complete inhibition at a concentration of 40 μM of collagen-induced platelet maximal aggregation. In platelet activation by ADP and epinephrine, allicin had already demonstrated significant inhibition of platelet maximal aggregation at concentrations of 5 and 10 μM (Fig. 1C). Testing the effect of various concentrations of allicin on a washed platelet aggregation slope demonstrated a sharp decline with complete inhibition at a concentration of 20 μM in ADP and epinephrine-activated platelets and a linear decrease in the slope with maximal inhibition at 40 μM in collagen-activated platelets (Fig. 1D). Addition of human serum albumin (50 mg/ml) to washed platelets completely abolished the inhibitory effect of allicin (40 μM) on platelet aggregation induced by ADP and epinephrine (data not shown).These