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Regular Article

Calmodulin antagonists induce platelet apoptosis

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ABSTRACT

Calmodulin (CaM) antagonists induce apoptosis in various tumor models and inhibit tumor cell invasion and metastasis, thus some of which have been extensively used as anti-cancer agents. In platelets, CaM has been found to bind directly to the cytoplasmic domains of several platelet receptors. Incubation of platelets with CaM antagonists impairs the receptors-related platelet functions. However, it is still unknown whether CaM antagonists induce platelet apoptosis. Here we show that CaM antagonists *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7), tamoxifen (TMX), and trifluoperazine (TFP) induce apoptotic events in human platelets, including depolarization of mitochondrial inner transmembrane potential, caspase-3 activation, and phosphatidylserine exposure. CaM antagonists did not incur platelet activation as detected by P-selectin surface expression and PAC-1 binding. However, ADP-, botrocetin-, and α-thrombin-induced platelet aggregation, platelet adhesion and spreading on von Willebrand factor surface were significantly reduced in platelets pre-treated with CaM antagonists. Furthermore, cytosolic Ca^{2+} levels were obviously elevated by both W7 and TMX, and membrane-permeable Ca^{2+} chelator BAPTA-AM significantly reduced apoptotic events in platelets induced by W7. Therefore, these findings indicate that CaM antagonists induce platelet apoptosis. The elevation of the cytosolic Ca^{2+} levels may be involved in the regulation of CaM antagonists-induced platelet apoptosis.
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Introduction

Calmodulin (CaM), an intracellular 17-kDa calcium-sensing protein, mediates the effects of changes in cytoplasmic Ca^{2+} concentration, and is also involved in the control of cytoplasmic Ca^{2+} concentration by regulating the activity of some Ca^{2+}-regulated proteins [1–5]. Binding of Ca^{2+} produces a conformational change in CaM, promoting interactions of the Ca^{2+}/CaM complex with numerous target proteins, thus regulating biological processes such as cell proliferation, vesicular fusion, and fertilization [1,6,7]. In particular, CaM has been shown to play roles in regulating apoptosis [3,8]. CaM antagonists induce apoptosis in various tumor models and inhibit tumor cell invasion and metastasis, thus some of which have been extensively used as anti-cancer agents [3,4,6,9]. For example, tamoxifen (TMX), a CaM antagonist and also a protein kinase C (PKC) inhibitor, has been in the center of management of hormone-sensitive breast cancer [4,10]. However, TMX has some severe side effects [11,12], one of which is thrombocytopenia [11,13]. Up to now, the pathogenesis of thrombocytopenia still remains unclear.

In platelets, CaM has been found to bind directly to the cytoplasmic domains of several platelet receptors [14–16]. Incubation of platelets with CaM antagonists impairs the receptors-related platelet function [14–16]. However, it is still unclear whether CaM antagonists induce platelet apoptosis. Apoptosis-like events were observed in platelets almost ten years ago [17,18]. The following studies verified that, like nucleate cells, platelet apoptosis induced by physiological [19] or chemical [20,21] compounds, or platelet storage [22,23] occurred widely *in vivo* and *in vitro*. Up to now, most of the platelet apoptotic events appear to be arisen from the mitochondrial pathway, characterized by depolarization of mitochondrial inner transmembrane potential (ΔΨm), caspase-3 activation, and verifications of Bcl-2 family protein expression [19–23]. Furthermore, phosphatidylserine (PS) exposure also occurs during platelet apoptosis induced by strong agonists such as A23187 [18], thrombin [19], or platelet storage under blood banking conditions [22,23]. Although some of the platelet apoptotic events are similar to those occurring in platelet activation, the signaling cascades leading to platelet apoptosis are distinct from those of platelet activation [24,25]. Platelets play a central role in maintaining integrity of endothelium and biological hemostasis. Platelet apoptosis would result in clearance of circulatory platelets, thus to explore the mechanisms underlying platelet apoptosis has important implications for thrombosis and hemostasis.

Various aromatic molecules have been widely used as CaM antagonists. *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide

Abbreviations: CaM, calmodulin; VWF, von Willebrand factor; TMX, tamoxifen; PS, phosphatidylserine; GP, glycoprotein; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide; BIM, Bisindolylmaleimide I hydrochloride; W5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; ΔΨm, mitochondrial inner transmembrane potential; TFP, trifluoperazine; TMRE, tetramethylrhodamine ethyl ester; PMA, phorbol 12-myristate-13-acetate; PKC, protein kinase C; PRP, platelet-rich plasma.

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(W7), one of naphthalenesulfonamide derivatives, inhibits cell proliferation [26], and also elicits platelet GPIbα ectodomain shedding [27]. Cationic derivatives of phenyl-substituted thiazole present the potential to be developed into anti-CaM drugs [28]. The phenothiazine drugs such as TMX [29,30] and trifluoperazine (TFP) [31] are already used clinically as anti-cancer and anti-psychotic agents. In the current study, we show that CaM antagonists W7, TFP and TMX, induce apoptotic events in human platelets.

Materials and methods

Reagents

Purified human von Willebrand factor (VWF) and botrocetin were generous gifts from Dr. Xiaoping Du (University of Illinois, Chicago, IL). Monoclonal antibodies SZ2 against GPIbα and SZ51 against P-selectin were generous gifts from Dr. Changgeng Ruan (Soochow University, Suzhou, China). W7 and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5) were purchased from Calbiochem (San Diego, CA). TMX, TFP, Bisindolylmaleimide I hydrochloride (BIM), ADP, phorbol 12-myristate-13-acetate (PMA), anti-human gelsolin antibody, and FITC-conjugated PAC-1 were purchased from Sigma (St. Louis, Missouri). 1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) was purchased from Dojindo Molecular Technologies (Rockville, MD). Monoclonal antibodies against Bax, Bak, Bcl-2 and caspase-3, FITC-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, California). FITC-conjugated annexin V and tetramethylrhodamine ethyl ester (TMRE) were from Bender Medsystem (Vienna, Austria). Gaot anti-mouse IgG-488 and Fluo-3/AM were purchased from Invitrogen/Molecular Probes (Invitrogen, USA).

Preparation of washed platelets and PRP

Fresh blood from healthy volunteers was anti-coagulated with 1/7 volume of acid-citratedextrose (ACD, 2.5% trisodium citrate, 2.0% D-glucose, 1.5% citric acid). After centrifugation, isolated platelets were washed twice with CGS buffer (0.123 M NaCl, 0.033 M D-glucose, 0.013 M trisodium citrate, pH 6.5) and resuspended in modified Tyrode's buffer (2.5 mM Hepes, 150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose, pH 7.4) to a final concentration of 3×10^8 /ml. In platelet-rich plasma (PRP) experiments, fresh blood was anti-coagulated with 1/9 volume of 3.8% trisodium citrate, and centrifuged at 150×g for 12 minutes (min) at room temperature (RT) to get PRP. Then, washed platelets and PRP were incubated at RT for 1 hour (h) to recover to resting state as described previously [32,33].

ΔΨm measurement assay

ΔΨm was determined using the potential sensitive dye tetramethylrhodamine-ethylester (TMRE). Briefly, washed platelets (3×10^8 /ml) were incubated with various concentrations of W7 (25 µM, 50 µM, 100 µM), TFP (10 µM, 25 µM, 50 µM), or TMX (0.5 µM, 10 µM, 25 µM, 50 µM) at 37 °C for 30 min. W5 (100 µM) was used as a control for W7, DMSO was used as a vehicle control for TMX and TFP. After resuspension at a concentration of 5×10^7/ml, platelets were incubated with TMRE (100 nM). Then the samples were further incubated in the dark at 37 °C for 20 min, and analyzed by flow cytometry. TMRE signals were excited using a 488 nm krypton-argon laser line and its emissions were captured using filters at 625 nm. To study the effect of protein kinase C (PKC), washed platelets were pre-incubated with PKC inhibitor BIM (10 µM) at 37 °C for 20 min before treatment of W7 (100 µM), and then ΔΨm was detected with TMRE by flow cytometry. To evaluate the effect of BAPTA-AM on CaM antagonists-induced depolarization of ΔΨm in platelets, washed platelets were pre-incubated with BAPTA-AM (10 µM) at 37 °C for 20 min before treatment of W7 (100 µM), and then ΔΨm was detected with TMRE by flow cytometry.

SDS-PAGE and protein immunoblotting

Washed platelets (3×10^8/ml) were incubated with W7, TFP or TMX at various concentrations, or vehicle control at 37 °C for 30 min, and lysed with an equal volume of lysis buffer containing 0.1 mM E64, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1/100 aprotinin on ice for 30 min. The whole lysate was resolved by SDS-PAGE, and Western analyses were performed using the following conditions: caspase-3 (1:1000); gelsolin (1:2500); Bcl-2 (1:500); Bax (1:500); and Bak (1:500). To evaluate the effect of BIM or BAPTA-AM on CaM antagonists-induced caspase-3 activation, washed platelets were pre-incubated with BIM (10 µM) or BAPTA-AM (10 µM) at 37 °C for 20 min before treatment of W7 (200 µM). To study the effects of calpain inhibitor on gelsolin cleavage, the platelets were pre-incubated with 50 µM calpain inhibitor 1.

PS exposure assay

Washed platelets (3×10^8/ml) were incubated with W7, TFP or TMX at various concentrations, or vehicle control at 37 °C for 30 min, and then resuspended at a final concentration of 5×10^7/ml. Annexin V binding buffer was mixed with treated platelets and annexin V-FITC at a 50:10:1 ratio. Samples were gently mixed by rocking and incubated at RT for 15 min in the dark, and then analyzed by flow cytometry. To evaluate the effect of BIM or BAPTA-AM on CaM antagonists-induced PS exposure, washed platelets were pre-incubated with BIM (10 µM) or BAPTA-AM (10 µM) at 37 °C for 20 min before treatment of W7 (200 µM), and then PS exposure was detected by flow cytometry.

Platelet surface staining

Washed platelets (3×10^8/ml) were incubated with W7 (100 µM), W5 (100 µM), or A23187 (5 µM) at 37 °C for 30 min, and then resuspended at a final concentration of 5×10^7/ml. To evaluate P-selectin surface expression, the platelets were incubated with anti-human P-selectin antibody (SZ51) at RT for 30 min. After washed once, platelets were further incubated with FITC-labeled anti-mouse IgG in the dark at RT for 30 min, and then analyzed by flow cytometry. For PAC-1 binding, the platelets were added into a FACS tube containing FITC-labeled soluble PAC-1, and incubated at RT for 20 min in the dark. Then, platelets were fixed with 1% cold paraformaldehyde, further incubated at 4 °C in the dark for 30 min, and analyzed by flow cytometry. A23187 was used as positive control. As negative control, platelets were incubated with mouse IgG and then incubated with FITC-labeled anti-mouse IgG.

Platelet aggregation

Platelet aggregation was performed using a turbidometric platelet aggregometer (Xinpusen, Beijing, China). Briefly, PRP or washed platelets were pre-incubated with W7 (100 µM), W5 (100 µM), or DMSO at 37 °C for 30 min, and then aggregation was induced by addition of botrocetin, ADP, PMA, or thrombin at 37 °C with a stirring speed of 1000 rpm.

Platelet spreading assay

Coverslips were coated with 100 µg/ml VWF at 4 °C overnight, washed three times with phosphate-buffered saline (PBS, pH 7.4), and blocked with heat-denatured bovine serum albumin (BSA, 5 mg/ml) at RT for 2 h. Washed platelets (5×10^7/ml) treated with W7 (100 µM), W5 (100 µM), or DMSO were applied to the coated coverslips in the presence of botrocetin (1 µg/ml) and incubated at

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