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ORIGINAL ARTICLE

Thrombin induces activation and translocation of Bid, Bax and Bak to the mitochondria in human platelets

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Summary. *Background:* Thrombin is a physiological platelet agonist that activates apoptotic events, including cytochrome *c* release and phosphatidylserine exposure; however, the mechanisms underlying these events remain unclear. *Objectives:* The present study is aimed to investigate whether thrombin induces activation and mitochondrial translocation of Bid, Bax and Bak. *Methods:* Changes in the mitochondrial membrane potential were registered using the dye JC-1; Bid, Bax and Bak translocation to the mitochondria was detected by immunoprecipitation and Western blotting in samples from mitochondrial and cytosolic fractions. *Results:* Treatment of platelets with thrombin or ADP induces activation and mitochondrial association of active Bid, Bax and Bak. Translocation of Bid and Bax to the mitochondria was reduced by cytochalasin D, latrunculin A or jasplakinolide. Platelet exposure to exogenous H_2O_2 (10 μM) results in activation of Bid and Bax, which was found to be similar to the effect of thrombin. Thrombin evokes mitochondrial membrane depolarization, which is attenuated by catalase. *Conclusion:* Our results indicate that thrombin induces activation and mitochondrial translocation of Bid, Bax and Bak, which is likely to be one of the apoptotic events in human platelets.

Keywords: apoptosis, Bax, Bid, hydrogen peroxide, mitochondria, platelets, thrombin.

Introduction

Apoptosis or programmed cell death is a well-conserved physiological pathway. A number of cellular agonists induce apoptotic events in platelets both *in vivo* and *in vitro* models [1–4]. Thrombin stimulates mitochondrial membrane potential depolarization, cytochrome *c* release and caspases-3 and -9 activation [4–6]. Platelet activation and apoptosis are activated by different concentrations of thrombin: low concentrations result in platelet activation but no apoptosis, while high concentrations induce apoptosis in a number of platelets [7]. Thrombin induces endogenous generation of H_2O_2, which stimulates cytochrome *c* release, caspase-3 and -9 activation and phosphatidylserine exposure [5]. Apoptosis in platelets induces the release of microparticles, which might play a role in the development of cardiovascular diseases [8]. A recent study has provided evidence supporting the role of apoptosis, and especially the antagonistic balance between Bcl-xL and Bak, in the determination of platelet life span [9].

Platelets express several components of the apoptotic machinery, including the proapoptotic proteins Bid and Bax [4,10]. Bid is a member of the 'BH3 domain only' subgroup of the Bcl-2 family proposed to connect surface death receptors with Bcl-2 or Bax [11]. In hepatocytes Bid is important for cytochrome *c* release, dysfunction of mitochondria and even cell death following Fas activation *in vivo*; however, other cell types do not require Bid for cytochrome *c* release [12]. Active Bax translocates to mitochondria where it inserts as an apparent homo-oligomerized integral membrane protein [13]. Bax and Bid have been suggested to induce permeabilization of the outer mitochondrial membrane, releasing multiple intermembrane space proteins [14]. Translocation of Bax to the mitochondria has been reported in response to oxidative stress [15,16]. Human platelets produce and release reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), under physiological stimulation [17–19] and in pathological situations, such as diabetes and ischemia/reperfusion [20,21]. Therefore, we have investigated the effect of physiological agonists on mitochondrial association of Bid, Bax and Bak and the involvement of endogenous H_2O_2 generation in human platelets.

Material and methods

Materials

Jasplakinolide (JP), Alexa Fluor 568-conjugated secondary antibody and MitoTracker Red were from Invitrogen (Madrid, Spain). Apyrase (grade VII), aspirin, thrombin, hydrogen

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peroxide, catalase, rotenone, fluorescein isothiocyanate (FITC)-conjugated phalloidin, monoclonal anti-Bax antibody (clone 6A7), anti-G-actin antibody and BSA were from Sigma (Madrid, Spain). Cytochalasin D (CytD) and latrunculin A were from Calbiochem (Nottingham, UK). Horseradish peroxidase-conjugated rabbit anti-sheep IgG antibody was from Santa Cruz (Santa Cruz, CA, USA). Protein A-agarose and anti-Bak antibody were from Upstate Biotechnology Inc. (Madrid, Spain). Anti-Bid antibody was from Cell Signaling (Barcelona, Spain). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) and Hyperfilm ECL were from Amersham (Arlington Heights, IL, USA). Anti-CoxIV antibody was from BD Transduction Laboratories (Madrid, Spain). Hirudin was from RayBiotech, Inc (Norcross, GA, USA). Enhanced chemiluminiscence detection reagents and mitochondria isolation kit were from Pierce (Cheshire, UK). All other reagents were of analytical grade.

Platelet preparation

Platelet suspensions were prepared as previously described [22], as approved by local ethical committees and in accordance with the Declaration of Helsinki. Briefly, blood was obtained from healthy drug-free volunteers and mixed with acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma (PRP) was prepared by centrifugation for 5 min at $700 \times g$ and aspirin (100 μM) and apyrase (40 μg mL^{-1}) were added. Cells were collected by centrifugation at $350 \times g$ for 20 min and resuspended in HEPES-buffered saline (HBS), pH 7.45, containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$ and supplemented with 0.1% BSA and 40 μg mL^{-1} apyrase. Final platelet concentration was 2×10^8 cells mL^{-1} unless otherwise stated.

Subcellular fractionation

Subcellular fractionation was performed using a commercial Mitochondria Isolation Kit (Pierce Biotechnology, Inc., Pierce, Rockford, IL, USA). Briefly, platelets were resuspended in the Mitochondria Isolation Reagent A and lysed with Mitochondria Isolation Reagent B at 4 °C. Samples were further mixed with Mitochondria Isolation Reagent C and then centrifuged at $700 \times g$ for 10 min at 4 °C. The supernatant was further subjected to centrifugation at $12\,000 \times g$ for 15 min to yield the mitochondrial pellet and the mitochondria-free cytosolic fraction (supernatant).

Inmunoprecipitation and Western blotting

Inmunoprecitation and Western blotting were performed as described previously [23]. Bax activation and Bax and Bid detection in mitochondria-free cytosolic fraction were detected by inmunoprecipitation. Briefly, 500 μL aliquots of platelet suspension and mitochondria-free cytosolic fraction were lysed and immunoprecipitated by incubation with 2 μg of anti-Bax antibody clone 6A7 or anti-Bid antibody and 25 μL of protein A-agarose overnight at 4 °C on a rocking platform. Proteins were separated by 15% SDS-PAGE and electrophoretically transferred, for 2 h at 0.8 mA cm^{-2}, in a semi-dry blotter (Hoefer Scientific, Newcastle, UK) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Membranes were incubated with the anti-Bax antibody diluted 1:500 in TBST for 2 h or with the anti-Bid antibody diluited 1:500 overnight. To detect the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:10 000 in TBST and exposed to enhanced chemiluminescence reagents for 5 min. Blots were exposed to photographic films and the optical density was estimated using scanning densitometry. For protein loading control, membranes containing whole cell lysates were reprobed with the anti-G-actin or the anti-CoxIV antibody. For samples that require immunoprecipitation, cell lysates were split and one set of samples was used for immunoprecipitation while the other was used for the detection of G-actin or CoxIV by Western blotting.

Confocal microscopy

Cells were fixed using 3% paraformaldehyde (in PBS) for 10 min, and then permeabilized in PBS containing 0.025% (v/v) Nonidet P-40 detergent for 10 min at 4 °C. Samples were incubated with anti-Bax antibody, diluted 1:500 in TBST for 2 h, followed by incubation with Alexa Fluor 568-conjugated secondary antibody diluted 1:10 000 in TBST for 1 h, 500 nM MitoTracker Red for 20 min and 1 μM FITC-phalloidin for 30 min. Samples were examined using a Zeiss LSM 510 confocal microscope with excitation wavelength of 488 nm and emission at 568, 600 and 515 nm, respectively.

Determination of mitochondrial membrane potential

Changes in the mitochondrial membrane potential were registered using JC-1. Platelets were loaded with JC-1 by incubating the PRP with 10 μg mL^{-1} JC-1 at 37 °C for 10 min. Platelets were then centrifuged and resuspended in HBS. JC-1-loaded cells were excited at 488 nm, and emission was detected at 585 nm (JC-1 aggregates) and 516 nm (JC-1 monomers) using a spectrofluorimeter. Data are presented as emission ratios (585/516) [24]. Thrombin-induced changes in mitochondrial membrane potential were quantified as the integral of the decrease in JC-1 fluorescence ratio for 1 min after the addition of thrombin and presented as percentage of the effect evoked by 10 μM rotenone.

Statistical analysis

Data are presented as means ± SEM of all the experiments performed in each experimental protocol. Analysis of statistical

significance was performed using Student's *t*-test. $P < 0.05$ was considered to be significant for a difference.

Results

Time course and concentration dependent activation of Bid and Bax by thrombin

Bid activation was analyzed by Western blotting using a rabbit anti-Bid antibody, which detects both the cleaved (active) and native forms of Bid [25]. Active Bax was detected by immunoprecipitation with the anti-Bax antibody (clone 6A7), which reacts only with Bax in its conformationally active state, followed by Western blotting with the same antibody as described previously [26]. Thrombin (1 U mL^{-1}) caused rapid activation of Bid and Bax (Figs 1A and 2A), which was detectable and significant 1 min after stimulation, reaching 120% ± 5% and 114% ± 4% of control, respectively, which was maintained for at least 60 min, with an increase of 219% ± 9% and 200% ± 6% of control, respectively (Figs 1A and 2A). The level of active Bid or Bax relative to total was 0.32, 0.38, 0.49, 0.61 and 0.71 for Bid and 0.26, 0.37, 0.51, 0.59 and 0.72 for Bax after stimulation with thrombin for 1, 10, 30 and 60 min, respectively.

The effect of thrombin on Bid and Bax activation was also concentration-dependent. After treatment for 1 h with thrombin a detectable increase in Bid and Bax activity was

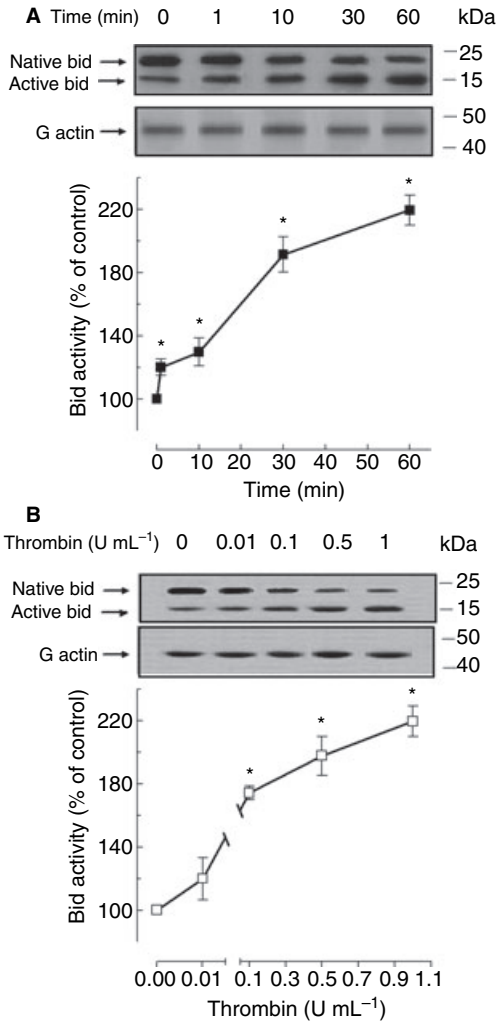

Fig. 1. Thrombin induces Bid activation. Platelets were treated either with 1 U mL^{-1} thrombin for various periods of time (A) or for 1 h with various concentrations of thrombin (0.01–1 U mL^{-1}; B) and then lysed. Samples were analyzed by Western blotting with the anti-Bid antibody followed by reprobing with the anti-G-actin antibody. $P < 0.05$ compared with control cells; $n = 6$.

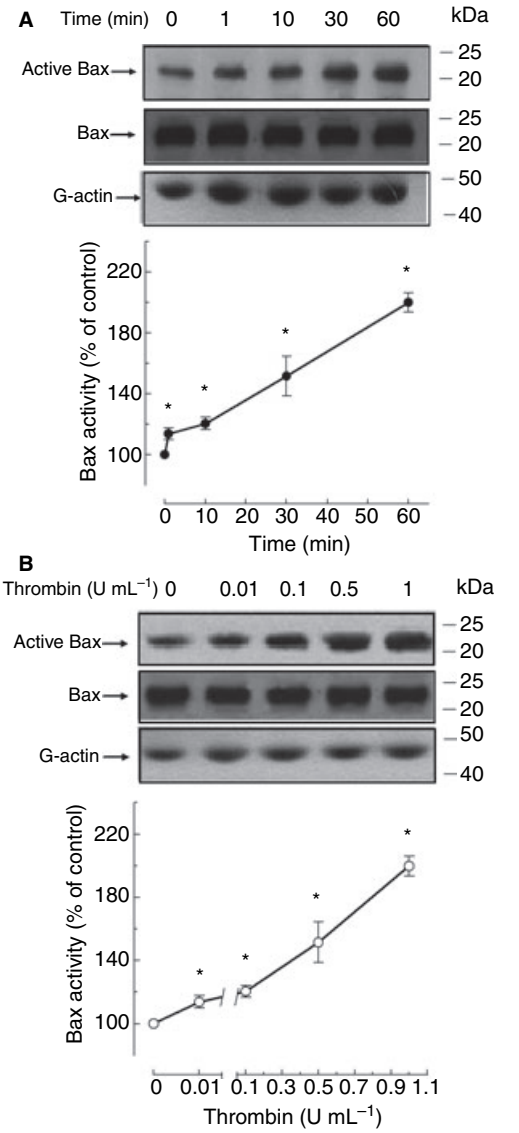

Fig. 2. Thrombin induces activation of Bax. Platelets were treated either with 1 U mL^{-1} thrombin for various periods of time (A) or for 1 h with various concentrations of thrombin (0.01–1 U mL^{-1}; B), lysed and split. One set of samples was immunoprecipitated with anti-Bax antibody (clone 6A7) and the immunoprecipitates were subjected to SDS/PAGE and Western blotting with the same antibody. Whole cell lysates from the second set of samples were analyzed by Western blotting with anti-Bax antibody and reprobed with the anti-G-actin antibody for protein loading control. $P < 0.05$ compared with control cells; $n = 6$.

observed at 0.01 U mL^{-1}; the effect was half maximal at 0.52 U mL^{-1}, and maximal at 1 U mL^{-1} (Figs 1B and 2B). The level of active Bid or Bax relative to total was 0.35, 0.46, 0.63, 0.69 and 0.73 for Bid and 0.21, 0.41, 0.53, 0.62 and 0.73 for Bax after stimulation with thrombin at 0.01, 0.1, 0.5 and 1 U mL^{-1}, respectively. Reprobing of the membranes with anti-G-actin antibody reported a similar amount of protein in all lanes (Figs 1 and 2).

Thrombin evokes translocation of active Bid and Bax to the mitochondria

Thrombin (1 U mL^{-1}) increases the mitochondrial association of active Bid and Bax, as detected by Western blotting with the anti-Bid antibody or by immunoprecipitation and subsequent Western blotting of samples of the mitochondrial fraction with anti-Bax antibody. Thrombin enhanced the amount of active Bid and Bax in the mitochondria by 212% ± 25% and 200% ± 22% of control, respectively (Fig. 3A and C). These results were confirmed by detection of labelled Bax and mitochondria by confocal microscopy (Fig. 3D). As a consequence, detection of Bid and Bax in the cytosol was significantly reduced (58% ± 11% and 76% ± 1% of control, respectively; Fig. 3A and C). The effect of thrombin was abolished by previous incubation with 3 antithrombin units (ATU) mL^{-1} hirudin for 10 min (Fig. 3B and E). SFLLRN (10 μM) and ADP (10 μM) also induced translocation of Bid and Bax to the mitochondria (Fig. 4A–D). In contrast, stimulation with the thromboxane A2 analogue, U46619, was unable to stimulate mitochondrial association of Bid or Bax (Fig. 4E and F). Detection of CoxIV (for the mitochondrial fraction) and G-actin (for the cytosolic fraction) revealed a similar amount of proteins in all lanes (Fig. 4).

The amount of Bid and Bax in the mitochondria stimulated by thrombin was reduced by treatment with CytD, an inhibitor of actin polymerization, to 131% ± 9% and 115% ± 11% of control, respectively (Fig. 5A and B). As a consequence, detection of Bid and Bax in the cytosol was significantly enhanced in the presence of CytD (185% ± 31% and 172% ± 18% of control; Fig. 5A and B). Similarly, treatment with 3 μM latrunculin A, a structurally unrelated actin polymerization inhibitor, attenuated thrombin-induced translocation of Bax to the mitochondria to 108.9% ± 8% of control (Fig. 5C). We have further investigated whether stabilization of the actin network impairs translocation of Bax to the mitochondria using JP, which induces actin filament polymerization and stabilization into a layer beneath the plasma membrane in

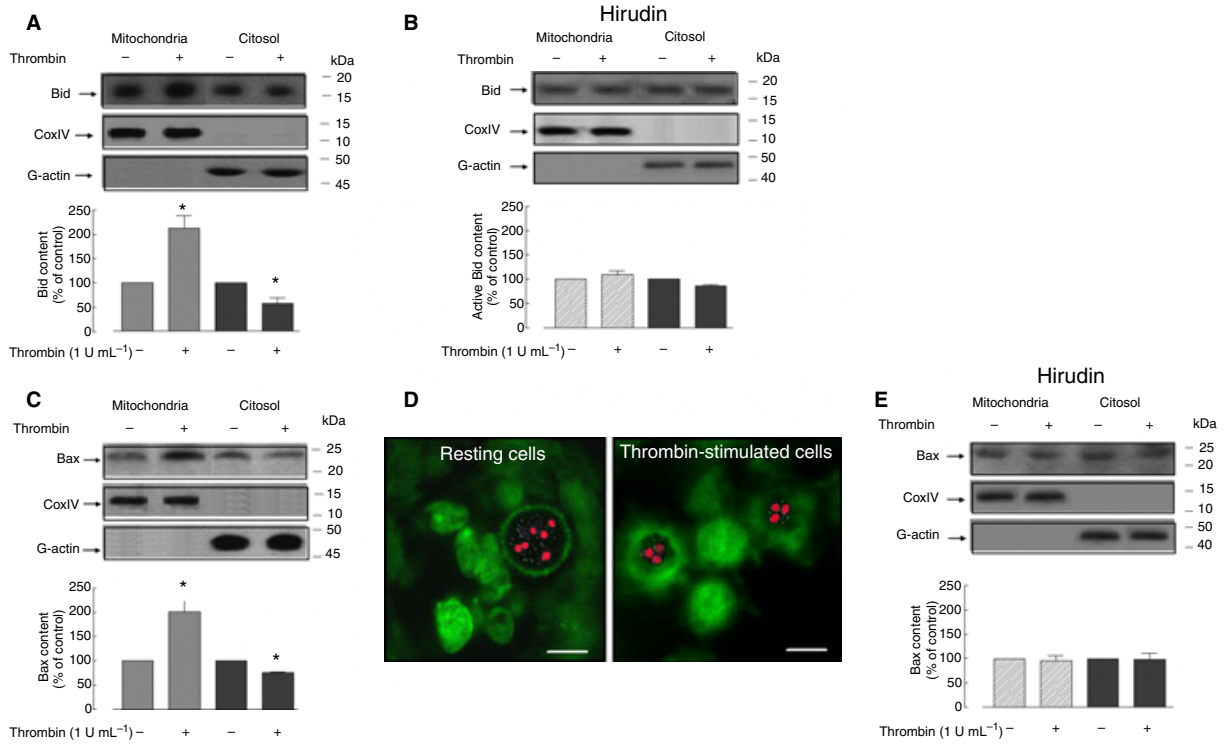

Fig. 3. Thrombin induces translocation of active Bid and Bax to the mitochondrial fraction. Platelets were treated with 1 U mL^{-1} thrombin for 1 h (A, C and D, right panel) or with thrombin 1 U mL^{-1} previously incubated with 3 ATU mL^{-1} hirudin for 10 min (B and E) and lysed. The mitochondrial and cytosolic fractions were isolated and active Bid and Bax detected as described in Material and methods. Data are presented as means ± SEM. $P < 0.05$ compared with control cells; $n = 6$. D, Confocal microscopy of human platelets immunostained with anti-Bax antibody followed by Alexa Fluor 568-conjugated secondary antibodies and stained with FITC-labelled phalloidin and MitoTracker Red. Left panel, control conditions. Right panel, platelets treated with 1 U mL^{-1} thrombin for 1 h. The bar represents 1 μm.

platelets [27]. As shown in Fig. 5D, treatment for 30 min with JP reduced thrombin-evoked mitochondrial association of Bax to 107% ± 10% of control.

Thrombin evokes translocation of Bak to the mitochondria

Furthermore, thrombin increases the mitochondrial association of Bak as detected by Western blotting in samples of the mitochondrial and cytosolic fractions with anti-Bak antibody. As shown in Fig. 6, thrombin enhanced the amount of Bak in the mitochondria by 201% ± 4% of control. As a consequence, detection of Bak in the cytosol was significantly reduced (61% ± 1%).

H_2O_2 induces activation and translocation of Bid and Bax to the mitochondria

Platelet stimulation with thrombin results in endogenous H_2O_2 production that is involved in a number of physiological processes [19,28]. Treatment with 10 μM H_2O_2 for 1 h enhanced the activity of both Bid and Bax by 165% ± 10% and 176% ± 11% of control, respectively (Fig. 7A and B). In addition, treatment with H_2O_2 significantly increased the mitochondrial association of active Bid and Bax (187% ± 29% and 192% ± 6% of control, respectively; Fig. 7C and D). As a consequence, detection of active Bid and Bax in the cytosol was significantly reduced (52% ± 7% and 69% ± 14% of control).

Thrombin-evoked mitochondrial depolarization is attenuated by catalase

In a medium containing 1 mM Ca^{2+}, thrombin (1 U mL^{-1}) induced mitochondrial membrane depolarization as detected by the decrease in JC-1 fluorescence ratio (585/516) (Fig. 8A). Rotenone (10 μM), which dissipates the membrane potential, induced maximal decrease in JC-1 fluorescence (Fig. 8A). Pretreatment for 30 min with 300 U mL^{-1} catalase [19] reduced thrombin-induced decrease in JC-1 fluorescence by 70% (Fig. 8B). Platelet stimulation with 10 μM H_2O_2 alone or in combination with thrombin resulted in a change in mitochondrial membrane potential similar to that induced by thrombin alone (Fig. 8B). These findings suggest that H_2O_2 might mediate thrombin-evoked mitochondrial depolarization.

Because CytD attenuated thrombin-evoked translocation of Bax to the mitochondria, we tested its effect on mitochondrial depolarization. Treatment with CytD did not impair thrombin-evoked mitochondrial depolarization (Fig. 8C), suggesting that Bax is not essential for this process.

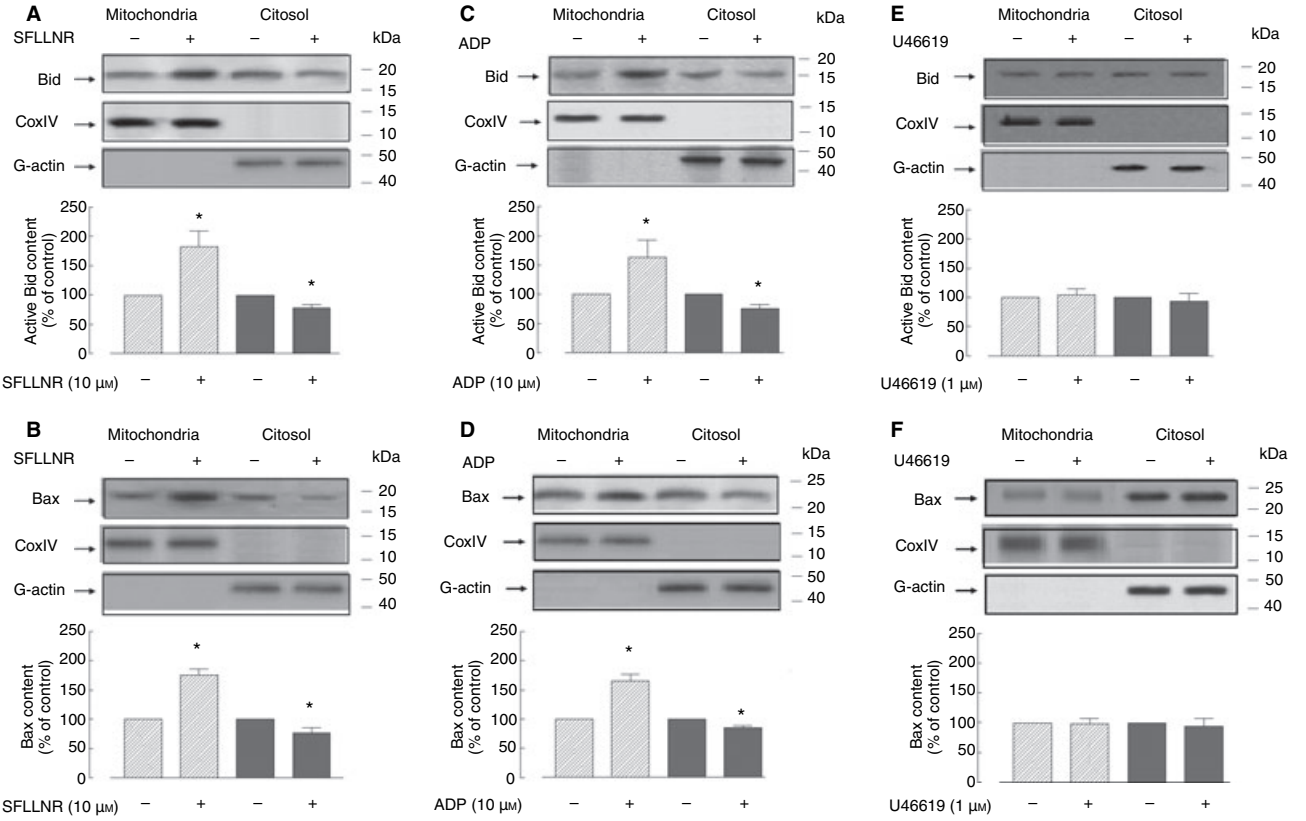

Fig. 4. Translocation of active Bid and Bax to the mitochondrial fraction stimulated by SFLLRN and ADP but not U46619. Platelets were stimulated with 10 μM SFLLRN, 10 μM ADP or 1 μM U46619 for 1 h and lysed. The mitochondrial and cytosolic fractions were isolated and active Bid and Bax detected as described in Material and methods. Data are presented as means ± SEM. $P < 0.05$ compared with control cells; $n = 6$.

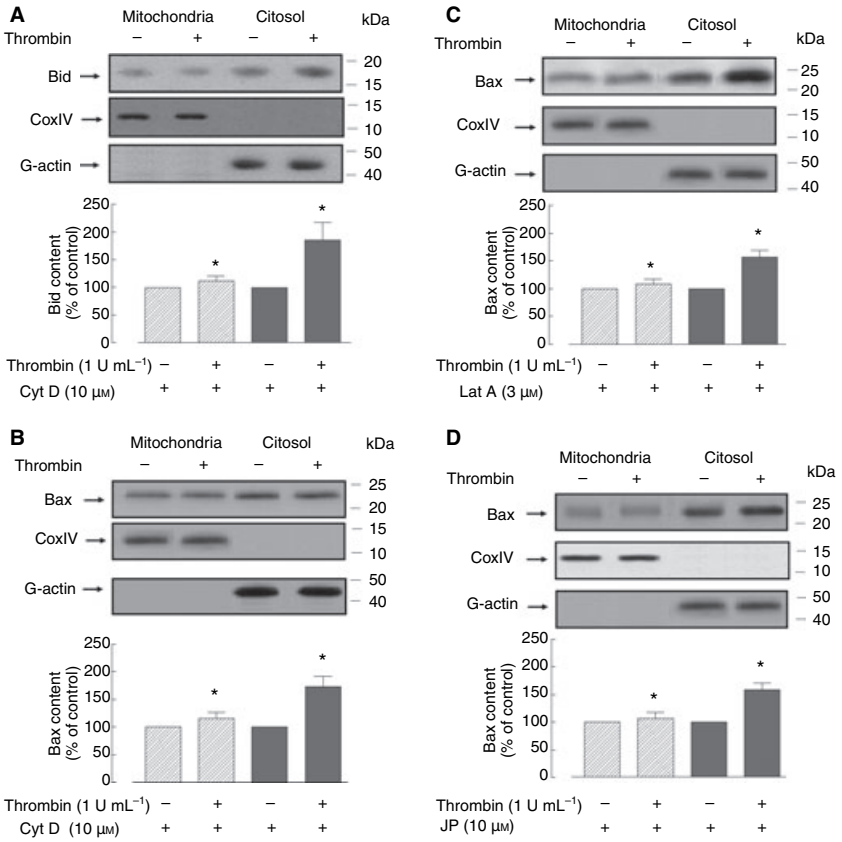

Fig. 5. Effect of cytoskeletal modifiers on thrombin-evoked translocation of active Bid and Bax to the mitochondrial fraction. Cells were incubated for 40 min with 10 μM CytD (A and B), for 1 h with 3 μM latrunculin A (C) or for 30 min with 10 μM JP (D), then treated with 1 U mL^{-1} thrombin for 1 h and lysed. The mitochondrial and cytosolic fractions were isolated and active Bid and Bax detected as described in Material and methods. Data are presented as means ± SEM. $P < 0.05$ compared with thrombin-treated cells (Fig. 3); $n = 6$.

Discussion

Cytochrome *c* release plays an essential role in apoptosis. One of the cellular mechanisms that mediate cytochrome *c* release involves mitochondrial swelling, either by opening of the permeability transition pore [29] or due to mitochondrial hyperpolarization [30]. However, apoptotic cells frequently contain unswollen mitochondria [31], and pro-apoptotic proteins, such as Bid or Bax, have been reported to release cytochrome *c* in the absence of detectable mitochondrial swelling [32,33]. In addition, the pro-apoptotic protein Bak has been reported to play a relevant role in platelet life span by the activation of programmed cell death [9]. Increased expression of proapoptotic Bax in platelets has been shown in response to serotonin [34], thrombin [35] and the Ca^{2+} ionophore ionomycin [36], as well as in aged platelets [37].

Here we describe for the first time activation and translocation of active Bid and Bax to the mitochondria upon platelet stimulation with the physiological agonists thrombin and ADP, but not with the thromboxane A2 analogue U46619, which induces 'apoptotic-like events' in platelets in the absence of phosphatidylserine externalization [38]. Upon thrombin stimulation the active forms of Bid and Bax are mostly located in the mitochondrial fraction, while the amount of the active proteins is reduced in the cytosol, thus confirming that the increase in the amount of active Bid and Bax in the mitochondrial fraction by thrombin is not due to activation of mitochondria-associated Bid and Bax. The effects of thrombin were mimicked by the PAR agonist SFLLRN and prevented by hirudin, which sugggests that this response was associated with the activation of thrombin receptors.

Platelets possess a dynamic actin cytoskeleton required for a number of cellular processes [39,40]. We have found that newly polymerized actin filaments are required for thrombin-induced translocation of active Bid and Bax to the mitochondria. This observation is in agreement with studies in HeLa cells [41] reporting that apoptosis triggers F-actin reorganization with an increase in the association with mitochondria. These findings suggest that the actin cytoskeleton might contribute to the initiation of apoptosis by enabling cytosolic proapoptotic proteins to be transported to mitochondria. Mitochondria are dynamic organelles in cells and the cytoskeleton has been reported to be important for the recruitment of mitochondria to enhance local Ca^{2+} buffering and energy supply [42]. In human platelets, where the treadmilling is very slow and treatment with CytD only prevents thrombin-induced actin polymerization without altering basal actin filament network [43], our results indicate

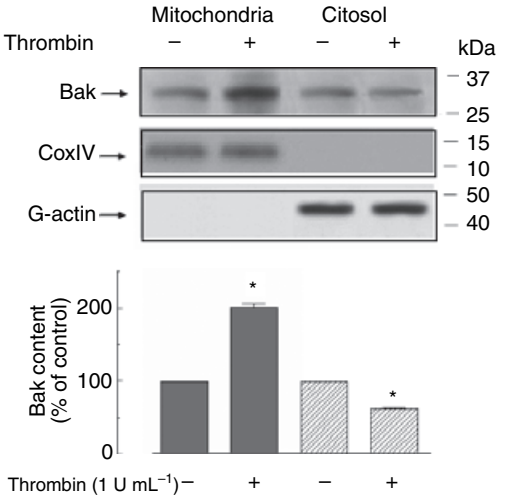

Fig. 6. Thrombin induces translocation of Bak to the mitochondrial fraction. Platelets were treated with 1 U mL^{-1} thrombin for 1 h and then lysed. The mitochondrial and cytosolic fractions were isolated and Bak detected as described in Material and methods. Data are presented as means ± SEM. $P < 0.05$ compared with control; $n = 6$.

that newly polymerized actin filaments are required for mitochondrial association of Bid and Bax upon platelet stimulation with thrombin, either for the movement of Bid and Bax to the mitochondria or for the dynamics of the mitochondrial network.

We have found that thrombin induces translocation of Bak to the mitochondria, which, as for Bid and Bax, is a process associated with Bak activation, thus suggesting that Bak is involved in thrombin-induced apoptotic events in human platelets, which might limit platelet life span in a number of cells during blood coagulation processes, when platelets are exposed to high concentrations of thrombin [7,9].

We have previously reported that thrombin is able to induce endogenous generation of H_2O_2 [19], which exerts different effects in human platelets, including Ca^{2+} mobilization, reduction of the activity of Ca^{2+}-ATPases, IP_3 receptor sensitization and inhibition of thrombin-induced aggregation [19,22,44]. Concerning apoptotic events in platelets, we have previously reported that exogenous H_2O_2 evokes cytochrome *c* release, caspase-3 and -9 activation and PS exposure [5]. Here we show that 10 μM H_2O_2, which is in the range of thrombin-evoked H_2O_2 generation in platelets [19], induces activation and mitochondrial translocation of Bid and Bax and mitochondrial depolymerization. In addition, catalase reduces thrombin-evoked mitochondrial depolymerization, thus suggesting that thrombin-evoked responses might be mediated by endogenous ROS generation as previously

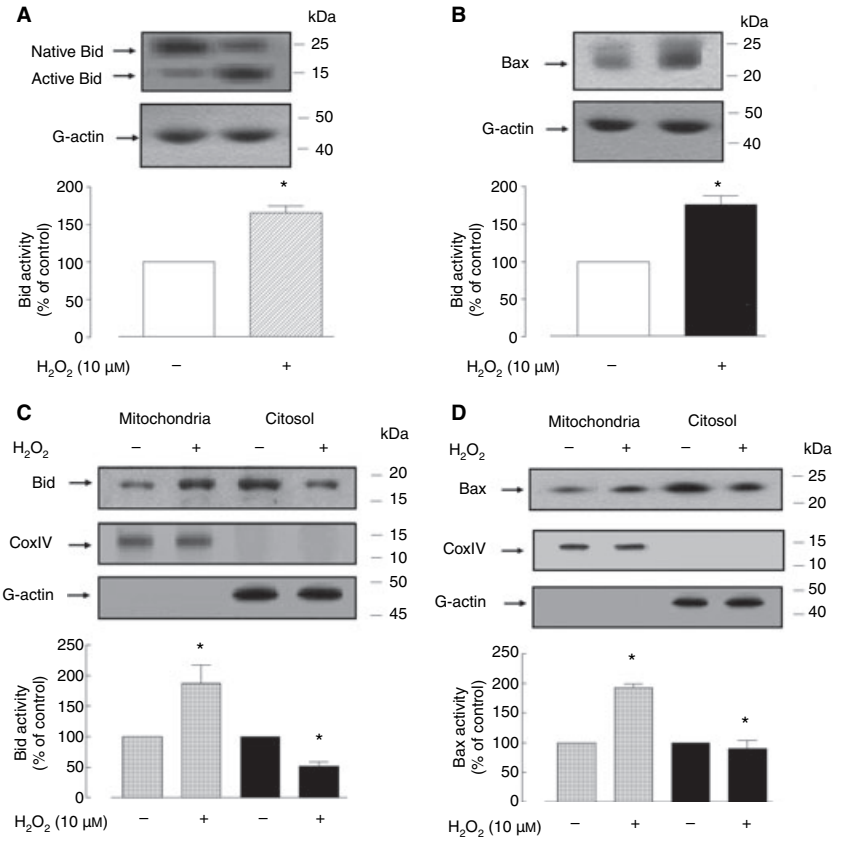

Fig. 7. H_2O_2 induces activation and translocation of active Bid and Bax to the mitochondrial fraction. Platelets were treated with 10 μM H_2O_2 for 1 h and then lysed. (A) Samples were subjected to SDS/PAGE and Western blotting with the anti-Bid antibody. (B) Samples were immunoprecipitated with anti-Bax antibody followed by Western blotting with the same antibody. (C and D) The mitochondrial and cytosolic fractions were isolated and active Bid (C) and Bax (D) detected as described in Material and methods. Data are presented as means ± SEM. $P < 0.05$ compared with control cells; $n = 6$.

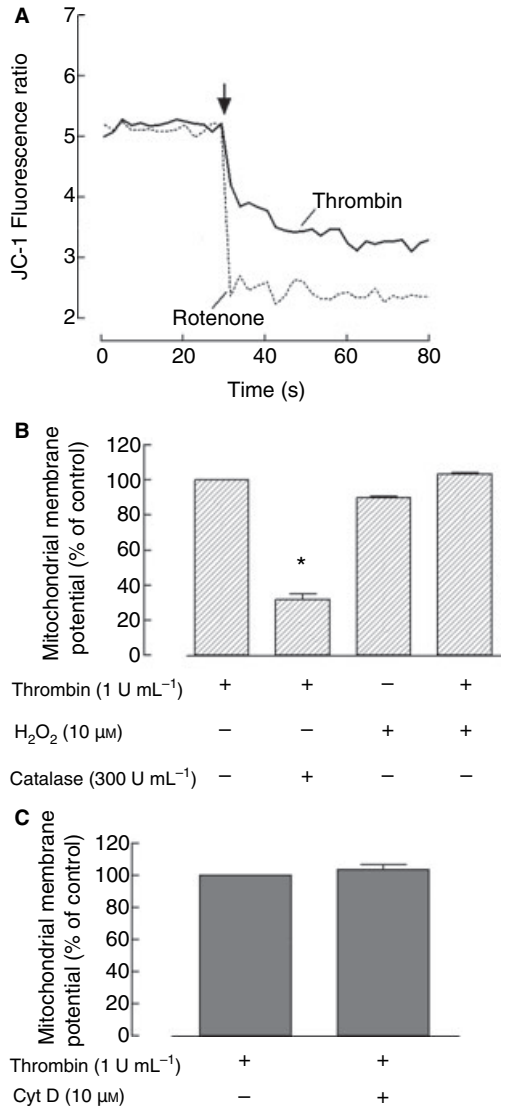

Fig. 8. Thrombin and H_2O_2 induce mitochondrial membrane depolarization. (A) JC-1-loaded cells were stimulated with 1 U mL^{-1} thrombin or 10 μM rotenone in a medium contaning 1 mM Ca^{2+}. (B) JC-1-loaded cells were preincubated for 30 min with 300 U mL^{-1} catalase or left untreated, as indicated. Cells were stimulated with 1 U mL^{-1} thrombin, 10 μM H_2O_2 or both. (C) JC-1-loaded cells were preincubated for 40 min with 10 μM CytD or left untreated, as indicated. Cells were stimulated with 1 U mL^{-1} thrombin. Histograms represent the changes in mitochondrial membrane potential relative to control (thrombin-stimulated cells). Data are presented as means ± SEM. $P < 0.05$ compared with control (thrombin-treated) cells; $n = 6$.

demonstrated for cytochrome *c* release [5]. In conclusion, our results provide evidence for the activation and mitochondrial translocation of Bid, Bax and Bak stimulated by thrombin in human platelets.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

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