

# Dual role of the p38 MAPK/cPLA<sub>2</sub> pathway in the regulation of platelet apoptosis induced by ABT-737 and strong platelet agonists

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p38 Mitogen-activated protein (MAP) kinase is involved in the apoptosis of nucleated cells. Although platelets are anucleated cells, apoptotic proteins have been shown to regulate platelet lifespan. However, the involvement of p38 MAP kinase in platelet apoptosis is not yet clearly defined. Therefore, we investigated the role of p38 MAP kinase in apoptosis induced by a mimetic of BH3-only proteins, ABT-737, and in apoptosis-like events induced by such strong platelet agonists as thrombin in combination with convulxin (Thr/Cvx), both of which result in p38 MAP kinase phosphorylation and activation. A p38 inhibitor (SB202190) inhibited the apoptotic events induced by ABT-737 but did not influence those induced by Thr/Cvx. The inhibitor also reduced the phosphorylation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), an established p38 substrate, induced by ABT-737 or Thr/Cvx. ABT-737, but not Thr/Cvx, induced the caspase 3-dependent cleavage and inactivation of cPLA<sub>2</sub>. Thus, p38 MAPK promotes ABT-737-induced apoptosis by inhibiting the cPLA<sub>2</sub>/arachidonate pathway. We also show that arachidonic acid (AA) itself and in combination with Thr/Cvx or ABT-737 at low concentrations prevented apoptotic events, whereas at high concentrations it enhanced such events. Our data support the hypothesis that the p38 MAPK-triggered arachidonate pathway serves as a defense mechanism against apoptosis under physiological conditions.

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Platelets play a key role in normal and pathological hemostasis through their ability to rapidly adhere to activated or injured endothelium and subendothelial matrix proteins (platelet adhesion), and to other activated platelets (platelet aggregation).<sup>1</sup> Platelets are also considered as key mediators of thrombosis, vascular inflammation, and atherosclerosis.<sup>2,3</sup>

Apoptosis play a significant role in platelet production from megakaryocytes, and circulating platelets contain many components of the apoptotic machinery.<sup>4</sup> Indeed, cytochrome c, caspase 9, caspase 3, apoptotic protease-activating factor, and Bcl-2 family proteins (for example, BAK, BAX, Bcl-X<sub>L</sub>, Bcl-2, Bcl<sub>w</sub>, Bim, Bid) are all expressed in platelets.<sup>5–8</sup>

A variety of stimuli can induce apoptotic or apoptotic-like events in platelets, such as ABT-737, thrombin, collagen, and A23187. ABT-737, a potent mimetic of Bcl-2 homology BH3-only proteins (including Bim, Bid, and other proteins that can be important for binding and neutralizing antiapoptotic Bcl-2 family proteins), induces caspase-dependent platelet apoptosis that is associated with BAX translocation from the cytosol to mitochondria and homo-oligomerization.<sup>7</sup> In highly activated platelets, several structural and functional changes, such as phosphatidylserine (PS) externalization, cell shrinkage, loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ), microparticle (MP) formation, and cleavage of gelsolin and

protein kinase C- $\delta$ , are reminiscent of nucleated cell apoptosis.<sup>9–13</sup> However, strong agonists either did not activate caspase 3<sup>14</sup> or did so very weakly.<sup>15–17</sup> A total of 13 types of cell death have been characterized for nucleated cells, including caspase-dependent and caspase-independent intrinsic apoptosis, autophagic cell death, necroptosis and others.<sup>18</sup> Although highly activated platelets exhibit the characteristic features of dying cells, there is no consensus in the literature clearly defining the type of strong activated platelet death. Some authors define them as necrotic cells<sup>19</sup> whereas others<sup>11,20,21</sup> consider them as apoptotic platelets.

Mitogen-activated protein (MAP) kinases represent a family of threonine/tyrosine-activated serine/threonine kinases that control many cellular responses, such as proliferation, migration, differentiation, and apoptosis. In platelets, p38 MAP kinase is phosphorylated and activated by different physiological agonists, including thrombin, collagen, and thromboxane A<sub>2</sub> (TxA<sub>2</sub>).<sup>22–25</sup> However, the mechanisms of p38 activation and their downstream effects are controversial and have not been clearly defined. p38 has been suggested to regulate platelet adhesion to collagen<sup>26</sup> and aggregation.<sup>24,27</sup> In contrast, using different p38 inhibitors, other authors did not find any significant effect of p38 on platelet activation.<sup>28–31</sup>

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**Abbreviations:** AA, arachidonic acid; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>;  $\Delta\Psi_m$ , mitochondrial membrane potential; MP, microparticle; ROS, reactive oxygen species; Thr/Cvx, thrombin/convulxin; TxA<sub>2</sub>, thromboxane A<sub>2</sub>; WP, washed platelets

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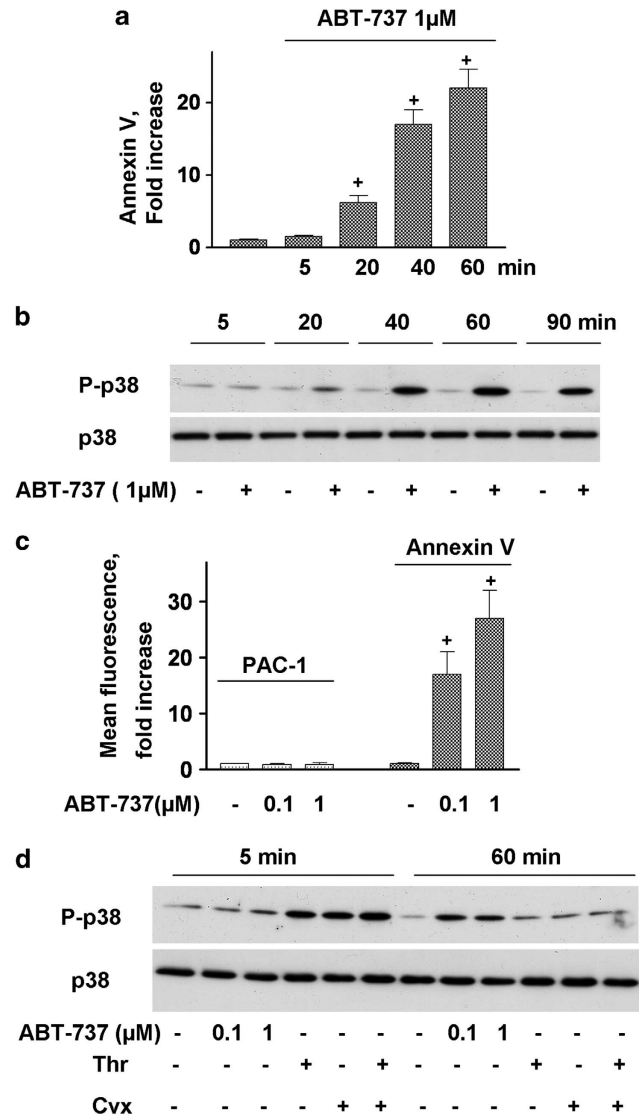
One of the established p38 substrates in platelets is cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), which is phosphorylated by p38 at serine505 in agonist-stimulated platelets.<sup>32–34</sup> In platelets, cPLA<sub>2</sub> activity is responsible for the release of arachidonic acid (AA) from membrane phospholipids.<sup>35</sup> AA is then metabolized by cyclooxygenase to prostaglandins, which are further converted to TxA<sub>2</sub> by thromboxane synthase (TxS). In addition, AA is involved in the regulation of reactive oxygen species (ROS) production. cPLA<sub>2</sub>-induced AA is a prominent requirement for the activation of NADPH oxidase and generation of ROS in phagocytes.<sup>36</sup> ROS may also be generated as a by-product during the oxidation of AA by cyclooxygenase or lipoxygenase.<sup>37,38</sup> Therefore, platelet cPLA<sub>2</sub> is involved in the regulation of two different functions, ROS generation and eicosanoid production. Excess ROS generation and AA release play essential roles in the initiation of apoptosis in platelets.<sup>21,39</sup> As p38/cPLA<sub>2</sub> is involved in the regulation of nucleated cell apoptosis<sup>40–43</sup> and the possible involvement of p38/cPLA<sub>2</sub> in the induction of platelet apoptotic events is not known, we compared the mechanism of p38/cPLA<sub>2</sub>-mediated signaling induced by platelet agonists and ABT-737.

In this study, we show that p38 plays a significant role in ABT-737-induced platelet apoptosis, whereas it has no effect on thrombin in combination with convulxin(Thr/Cvx)-induced apoptotic-like events. ABT-737, but not Thr/Cvx, induced the caspase-dependent cleavage and inactivation of cPLA<sub>2</sub>. We also assessed multiple influences of AA/ROS on apoptotic-like events in platelets and suggest that the p38-triggered arachidonate pathway serves as a defense mechanism under physiological conditions.

## Results

**Activation of p38 MAP kinase plays a significant role in ABT-737-induced platelet apoptosis.** To clarify the function of p38 in platelet apoptosis induced by two independent pathways, the BH3 mimetic compound ABT-737 was used as an activator of the BAK/BAX-caspase pathway and a combination of Thr/Cvx as agonists of the second caspase-independent pathway.

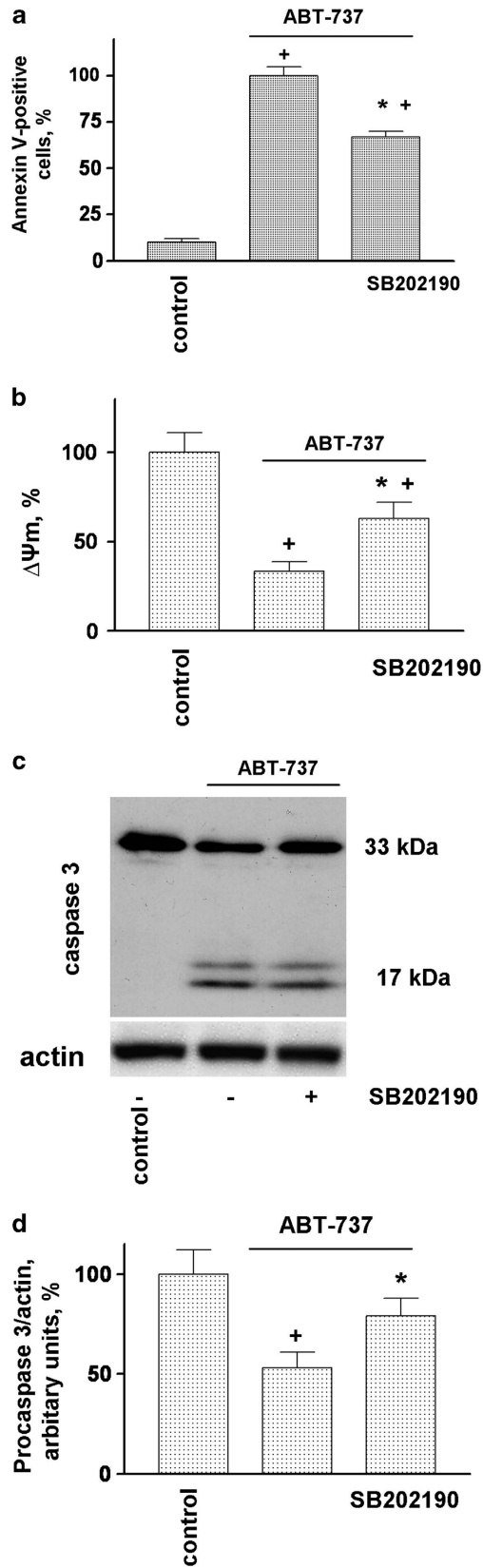
In our study, we used terms apoptosis-like events for platelets activated by strong agonists and apoptosis for ABT-737-induced platelet death. ABT-737 at concentrations ranging from 0.1 to 1  $\mu$ M induced dose- and time-dependent increases in Annexin V binding (Figures 1a and c). Simultaneously, ABT-737 (0.1–1  $\mu$ M) time dependently increased the phosphorylation of p38 (Figure 1b). In contrast to Thr/Cvx, ABT-737 did not activate  $\alpha$ IIb $\beta$ 3 integrins (Figure 1c) and did not induce MP formation (Figures 6a and b). The activation of p38 by thrombin and convulxin alone or in combination (Thr/Cvx) was transient, reaching a maximum after 5 min of incubation and then gradually decreasing to a level comparable with that of the control after 60 min (Figure 1d). Conversely, activation of p38 by ABT-737 was detectable only after 20 min of stimulation and remained constant even up to 90 min of stimulation (Figure 1a). A p38 inhibitor (SB202190, 1  $\mu$ M) significantly decreased the amount of Annexin V-positive platelets (67  $\pm$  3% compared with



**Figure 1** BH3 mimetic (ABT-737) phosphorylates p38. (a) FACS analysis of Annexin V binding and (c) PAC-1 binding (integrin  $\alpha$ IIb $\beta$ 3 activation, mean fluorescence, AU) to WP ( $1 \times 10^7$ /ml) incubated with ABT-737. Annexin V and PAC-1 binding are presented as the fold increase compared with the control taken as 1. Data are presented as means  $\pm$  S.E.M,  $n = 5$ ;  $+ P < 0.05$  compared with the control. (b and d) Western blot analysis using WP ( $4 \times 10^9$ /ml) of p38 phosphorylation after incubation with thrombin (5 mU/ml) and convulxin (5 ng/ml) or ABT-737 (0.1–1  $\mu$ M) (data shown are representative of three independent experiments)

ABT-737-stimulated platelets taken as 100%) and prevented decreases in  $\Delta\Psi_m$  ( $32 \pm 4\%$ ) stimulated by ABT-737 (Figures 2a and b). Similarly, SB202190 inhibited the cleavage of procaspase 3 by  $31 \pm 3\%$  (Figures 2c and d), indicating that p38 is partly involved in ABT-737-induced caspase 3 activation and apoptosis.

**p38 MAP kinase is not involved in the generation of apoptotic-like events triggered by Thr/Cvx.** We next investigated whether the activation of p38 is involved in Thr/Cvx-induced platelet death. The activation of platelets by Thr/Cvx significantly increased Annexin V binding  $7.6 \pm 1.5$



compared with control  $1 \pm 0.14$ ) and  $\alpha$ IIb $\beta$ 3 integrin activation (Figure 3a). The inhibition of p38 (SB202190,  $1 \mu\text{M}$ ), as assessed by the phosphorylation of its established substrate (HSP27) (Figures 3c and d), did not significantly inhibit Annexin V binding (Figure 3a) or decreases in  $\Delta\Psi_m$  (Figure 3b) and had no effect on  $\alpha$ IIb $\beta$ 3 integrin activation (Figure 3a), indicating that p38 is not involved in Thr/Cvx-induced platelet apoptotic-like events.

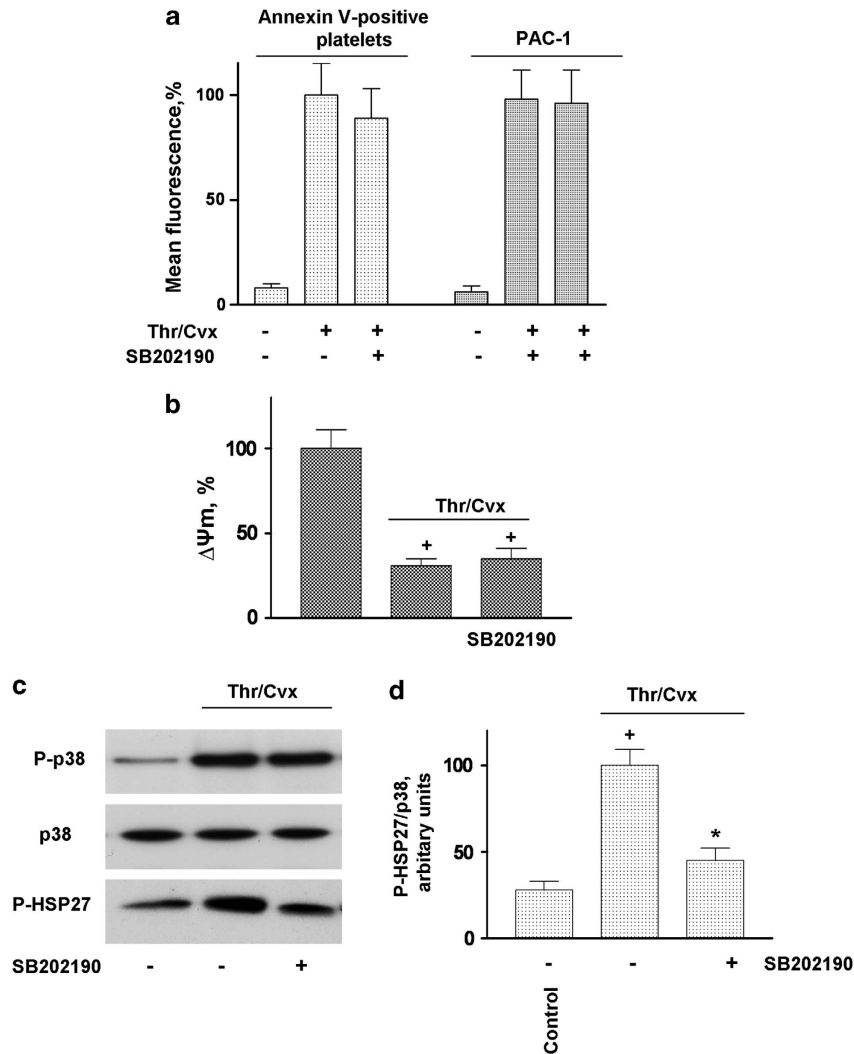
#### Platelet cPLA<sub>2</sub> cleavage by ABT-737 is caspase dependent.

The stimulation of platelets by Cvx and Thr/Cvx (both 5 min) and by ABT-737 (30 min) induced p38 and cPLA<sub>2</sub> phosphorylation, and the latter was partly (in the case of Cvx and Thr/Cvx) and fully (in the case of ABT-737) prevented by preincubation with SB202190 (Figures 4a and b). However, in contrast to Cvx and Thr/Cvx, ABT-737 induced the cleavage of cPLA<sub>2</sub>, which was prevented by a procaspase 3 inhibitor (z-DEVD-fmk) at concentrations of 50–100  $\mu\text{M}$  (Figures 4c and d). We then tested whether the cleavage of cPLA<sub>2</sub> would inhibit its activity. One of the main functions of cPLA<sub>2</sub> in platelets is associated with the production of thromboxane (TxA<sub>2</sub>) TxS. ABT-737 ( $1 \mu\text{M}$ ) induced only a weak activation of TxS ( $4 \pm 0.3$ -fold increase compared with the control taken as 1), Cvx and Thr/Cvx (Figure 4e) strongly activated TxS ( $20 \pm 1.3$ - and  $52 \pm 1.3$ -fold, respectively). The inhibition of p38 (SB202190,  $1 \mu\text{M}$ ) decreased the TxS activity induced by Cvx or Thr/Cvx to  $75 \pm 1.7\%$  and  $87 \pm 1.8\%$ , respectively, compared with the agonist-induced activity taken as 100% (Figure 4f).

#### Arachidonic acid prevents apoptosis in platelets at low concentrations and induces apoptosis in platelets at high concentrations.

In nucleated cells, AA has been shown to contribute to mitochondrial dysfunction during apoptosis;<sup>44,45</sup> however, AA was used at very high concentrations (more than  $10 \mu\text{M}$ ) in these studies. The depletion of AA prevents apoptotic development in cold-stored platelets whereas incubation with extremely high concentrations of AA (50–100  $\mu\text{M}$ ) induces platelet apoptosis.<sup>39</sup> In the present study, we tested whether AA would contribute to apoptosis in freshly isolated platelets. Washed platelets (WP) were incubated with different concentrations of AA ( $10^{-7}$  to  $10^{-4}$  M), and apoptosis was assessed by decreases in  $\Delta\Psi_m$ , PS exposure, and procaspase 3 cleavage. At high concentrations ( $10^{-5}$  and  $10^{-4}$  M), AA did indeed induce a decline in  $\Delta\Psi_m$  (Figure 5a) and an increase in Annexin V binding (Figure 5b) and cleavage of procaspase 3 after 24 h of incubation (Figure 5c). However, at low concentrations ( $10^{-6}$  and  $10^{-7}$  M), AA appeared to have antiapoptotic effects: it did not affect Annexin V binding, slightly increased

**Figure 2** p38 MAP kinase inhibition prevents ABT-737-induced platelet apoptosis (a) FACS analysis of Annexin V-positive platelets ( $1 \times 10^7/\text{ml}$ ) and (b) mitochondrial membrane potential ( $\Delta\Psi_m$ ) in WP incubated with ABT-737 ( $0.1 \mu\text{M}$ , 30 min) or pretreated (10 min,  $1 \mu\text{M}$ ) with p38 inhibitor (SB202190). (c) Western blot and (d) densitometry analyses of caspase 3 in human WP ( $4 \times 10^8/\text{ml}$ ) incubated with ABT-737 ( $0.1 \mu\text{M}$ ) or pretreated (10 min,  $1 \mu\text{M}$ ) with p38 inhibitor (SB202190). In (a), ABT-737 was taken as 100%; in (b) and (d), the control was taken as 100%. Data are presented as means  $\pm$  S.E.M.,  $n=4$ ; +  $P<0.05$  compared with the control, \* $P<0.05$  compared with ABT-737



**Figure 3** p38 inhibitor does not prevent PS surface exposure, decreased  $\Delta\Psi_m$ , and integrin  $\alpha IIb\beta 3$  activation in platelets stimulated with Thr/Cvx (a) FACS analysis of Annexin V-positive cells and PAC-1 binding and (b)  $\Delta\Psi_m$  in WP ( $1 \times 10^7$ /ml) stimulated by Thr/Cvx or pretreated (10 min,  $1 \mu\text{M}$ ) with p38 inhibitor (SB202190). In (a) and (d), Thr/Cvx was taken as 100%; in (b), control was taken as 100%. Data are presented as means  $\pm$  S.E.M,  $n=6$ ; +  $P < 0.05$  compared with the control, \*  $P < 0.05$  compared with platelets incubated with Thr/Cvx. (c) Western blot and (d) densitometry analyses of p38 and HSP27 phosphorylation in WP ( $3 \times 10^8$ /ml) stimulated with Thr/Cvx or pretreated (10 min,  $1 \mu\text{M}$ ) with p38 inhibitor (SB202190). The data shown are representative of three independent experiments

$\Delta\Psi_m$  (Figure 5a), and decreased the procaspase 3 cleavage (Figures 5d and e) induced by ABT-737 and procaspase 3 cleavage in stored platelets after 24 h (Figure 5c). At low concentrations, AA also decreased the Annexin V binding induced by Thr/Cvx and ABT-737. In accordance with these data, AA induced MP formation only at high concentrations (Figures 6a and b).

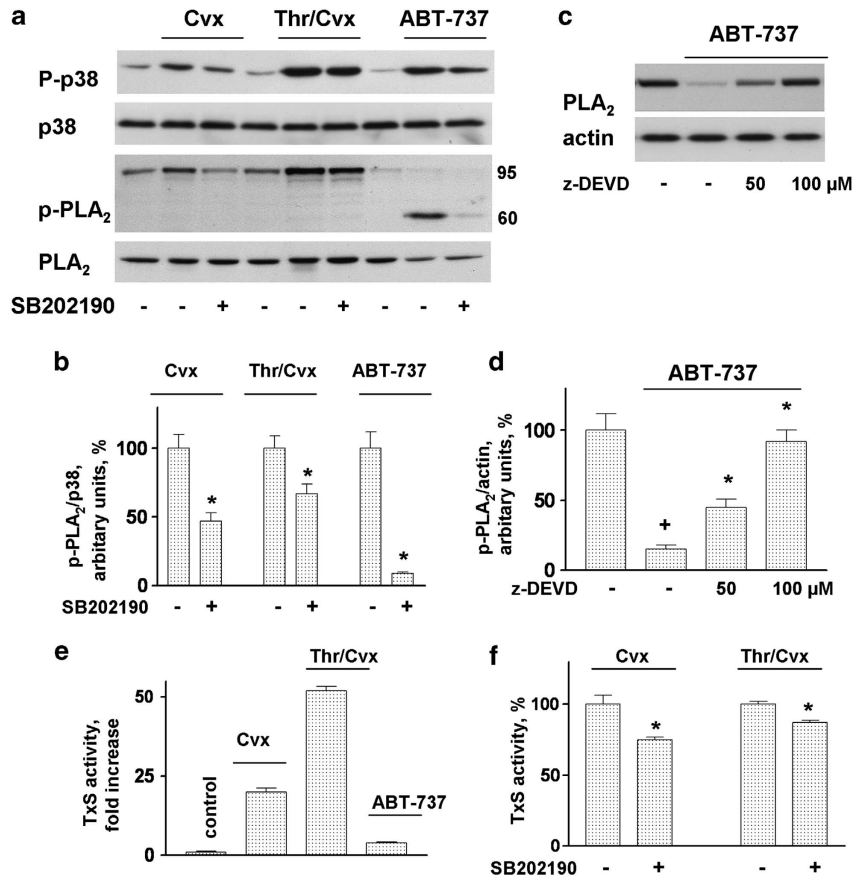
**ROS generated by activated platelets are not directly correlated with apoptotic events.** AA can be involved in the regulation of ROS production as a by-product during the oxidation of AA by cyclooxygenase or lipoxygenase.<sup>37</sup> In addition, AA can directly activate NADPH oxidase, inducing ROS generation.<sup>46</sup> As MP formation in platelets may be induced by ROS,<sup>47,48</sup> we compared MP formation and ROS production in platelets activated by ABT-737 ( $1 \mu\text{M}$ ), AA ( $10^{-6}$  and  $10^{-4}$  M), and Thr/Cvx (5 mU/5 ng/ml). AA at a concentration of  $10^{-4}$  M induced the highest amount of

MPs ( $2.7 \pm 0.34$  fold compared with the control taken as 1), and Thr/Cvx induced  $1.7 \pm 0.2$ ; neither a low concentration of AA ( $10^{-6}$  M) nor  $1 \mu\text{M}$  ABT-737 induced significant MP formation (Figures 6a and b).

ROS production was strongly induced by AA at a low but not at a high concentration ( $27 \pm 5.6$  and  $3 \pm 1.5$ -fold respectively, compared with the control taken as 1) (Figure 6c). Thr/Cvx stimulation also significantly increased ROS production ( $20 \pm 4$ -fold, compared with the control taken as 1), whereas ABT-737 had no effect on platelet ROS production (Figure 6c).

## Discussion

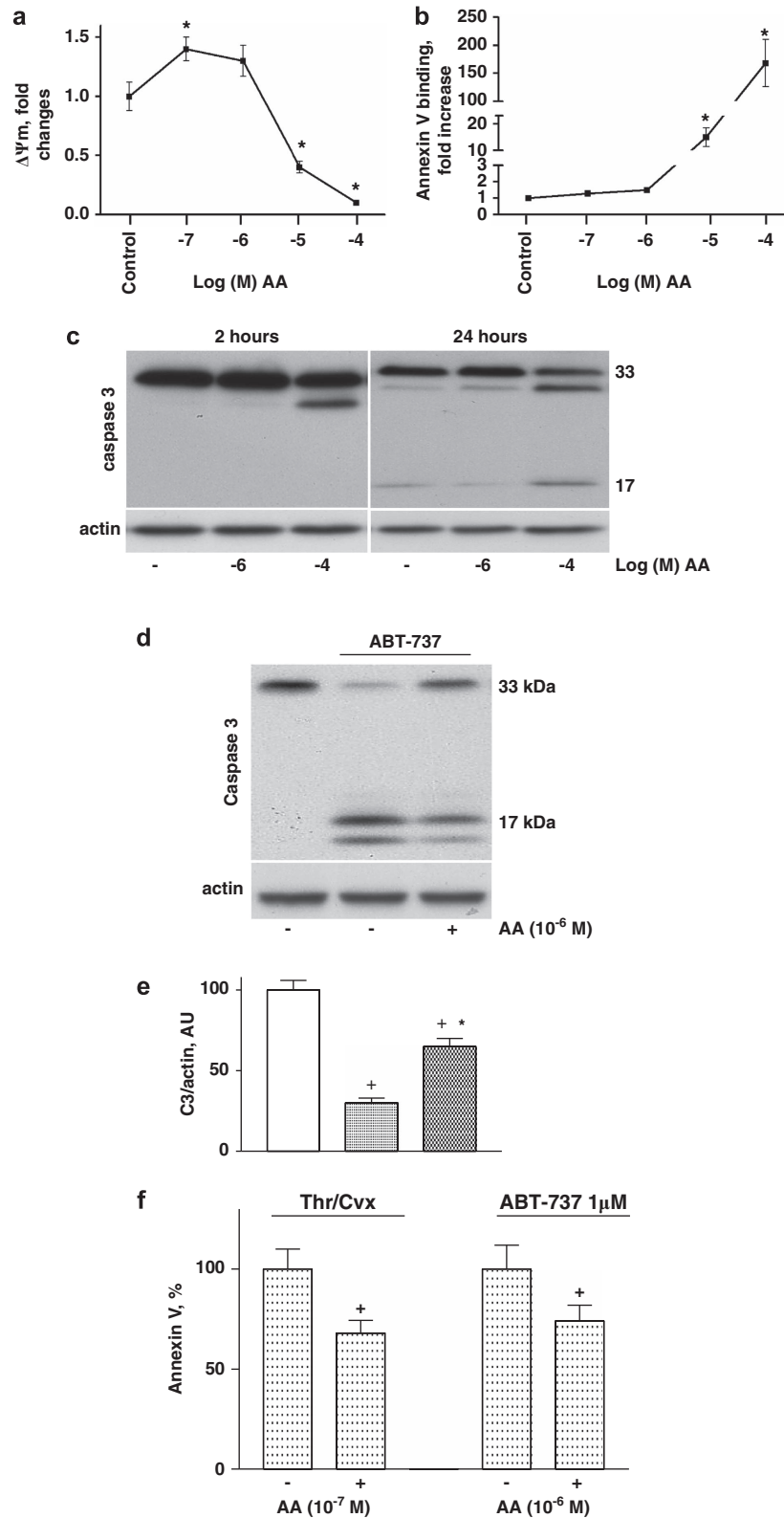
In nucleated cells, stress-activated p38 MAPK is involved in apoptosis, cytokine production, cytoskeleton reorganization, and transcriptional regulation. p38 MAPK also appears to play a role in the pathogenesis of heart ischemia, sepsis, arthritis,



**Figure 4** ABT-737 cleavage of platelet cPLA<sub>2</sub> is caspase dependent. (a) Western blot and (b) densitometry analysis of p-cPLA<sub>2</sub> in WP incubated with Cvx (5 ng/ml), Thr/Cvx (5 mU/5 ng/ml), or ABT-737 (1 μM) or pretreated (10 min, 1 μM) with p38 inhibitor (SB202190). (c) Western blot and (d) densitometry analysis of cPLA<sub>2</sub> in WP after stimulation with ABT-737 or pretreated with the indicated concentrations of the caspase 3 inhibitor:z-DEVD. Western blot data are representative of three independent experiments of p38 and cPLA<sub>2</sub> phosphorylation in WP (4 × 10<sup>9</sup>/ml). (e and f) ELISA data for the TxS activity in WP (4 × 10<sup>9</sup>/ml) activated by Cvx (5 ng/ml, 5 min), Thr/Cvx (5 mU/5 ng/ml, 5 min), or ABT-737 (1 μM, 30 min) and after preincubation with SB202190 (1 μM, 10 min)

human immunodeficiency virus infection, and Alzheimer's disease.<sup>49</sup> In platelets, p38 MAPK has been shown to be activated by almost all platelet agonists, including thrombin, collagen, ADP, and TxA<sub>2</sub>.<sup>22–25</sup> Nonetheless, the involvement of p38 in the mechanisms that trigger apoptosis in platelets has remained unclear. Therefore, we investigated the functions of p38 in the regulation of apoptosis induced by ABT-737 and the apoptosis-like events induced by strong agonists (Thr/Cvx). We found that ABT-737 dose- and time-dependently resulted in the phosphorylation of p38 (Figure 1). The inhibition of p38 significantly prevented platelet apoptosis, as assessed by PS-positive cells, a decrease in mitochondrial potential, and cleavage of caspase 3 (Figure 2). cPLA<sub>2</sub> is an established p38 substrate in platelets,<sup>23,32–34</sup> and we show that the ABT-737-induced activation of p38 triggered cPLA<sub>2</sub> phosphorylation. In nucleated cells undergoing apoptosis, cPLA<sub>2</sub> is cleaved and inactivated by caspase 3 activation.<sup>50,51</sup> In platelets, cPLA<sub>2</sub> was also cleaved upon ABT-737 activation and inactivated by a caspase 3-dependent mechanism (Figures 4a and b). In contrast, Thr/Cvx treatment induced the phosphorylation of cPLA<sub>2</sub> but did not promote its degradation (Figure 4a). Consistent with these data,

Thr/Cvx strongly enhanced cPLA<sub>2</sub> activity compared with the effect of ABT-737 (Figure 4e). It is important to note that the inhibition of p38 significantly prevented the apoptosis induced by ABT-737 but had no effect on the apoptotic-like events triggered by Thr/Cvx stimulation (Figure 3). As ABT-737-induced platelet apoptosis was directly correlated with the cleavage and inactivation of cPLA<sub>2</sub> and because Thr/Cvx stimulation strongly activated cPLA<sub>2</sub>, which was responsible for AA generation, we tested whether AA itself was involved in apoptosis. In nucleated cells, high concentrations of AA contribute to mitochondrial dysfunction and apoptotic cell death,<sup>44,45,52</sup> and AA also promotes apoptosis in stored platelets.<sup>39</sup> In addition, the concentration of AA can increase up to 10-fold during cerebral or heart ischemia compared with normal physiological conditions.<sup>53,54</sup> Therefore, we tested the effects of different AA concentrations on hallmarks of platelet apoptosis. AA at low concentrations (10<sup>-7</sup>–10<sup>-6</sup> M) enhanced ΔΨ<sub>m</sub>, but had no influence on PS externalization and did not cleave caspase 3. In contrast, AA at high concentrations (10<sup>-5</sup>–10<sup>-4</sup> M) induced a decrease in ΔΨ<sub>m</sub>, enhanced PS externalization, and activated caspase 3 (Figure 5). Thus, the amount of AA might be an essential



**Figure 5** Arachidonic acid at low concentrations prevents, and at high concentrations induces apoptosis in platelets. (a) FACS analysis of  $\Delta\Psi_m$  and (b) Annexin V binding in WP ( $1 \times 10^7$ /ml) stimulated with AA for 5 min at the indicated concentrations. (f) Inhibition of Annexin V binding by AA ( $10^{-7}$ – $10^{-6}$  M) in platelets stimulated with Thr/Cvx (5 min) and ABT-737 ( $1 \mu$ M, 30 min). (c and d) Western blot analysis (data shown are representative of three independent experiments) of caspase 3 in WP ( $4 \times 10^9$ /ml) after stimulation with AA at the indicated concentrations for 2 and 24 h (c) and after stimulation with ABT-737 alone and in combination with AA for 30 min (d) and (e) densitometry analysis of (d). In (e), the control was taken as 100%. Data are presented as means  $\pm$  S.E.M,  $n=6$ ; +  $P < 0.05$  compared with the control, \*  $P < 0.05$  compared with platelets incubated with ABT-737

factor for mediating pro or antiapoptotic-like effects in platelets. We next addressed whether different concentrations of AA could prevent or enhance the apoptotic events induced by Thr/Cvx and ABT-737. Accordingly, we found that AA at low concentrations ( $10^{-7}$  for Thr/Cvx and  $10^{-6}$  for ABT-737) inhibited the apoptotic events induced by both stimuli (Figure 5). The higher concentrations of AA required for the inhibitory effects of ABT-737-induced apoptosis were most likely associated with the inactivation of cPLA<sub>2</sub> and consequently a reduced basal AA concentration in this case.

ROS are involved in cellular responses to stress<sup>55</sup> and may contribute to various cellular pathological events, including apoptosis<sup>56</sup> and, in particular, MP formation.<sup>47,48</sup> We investigated whether MP formation induced by ABT-737, Thr/Cvx, and AA correlates with ROS production and found increases only in the Thr/Cvx- and  $10^{-6}$  M AA-treated platelets (Figure 6c), whereas MPs were enhanced only in the Thr/Cvx- and  $10^{-4}$  M AA-stimulated platelets (Figures 6a and b), indicating that ROS are most likely not directly linked to apoptotic events.

In summary, we show here that p38 MAP kinase plays a significant role in platelet apoptosis induced by ABT-737, but is not involved in the generation of the apoptotic-like events

triggered by physiological stimuli (Thr/Cvx). Similarly, under physiological conditions, the p38-triggered activation of cPLA<sub>2</sub>, release of AA, and ROS generation may serve as mechanisms to protect cells from apoptosis. In contrast, a high concentration of AA (as often observed under pathological conditions) may promote proapoptotic events.

## Materials and Methods

**Materials.** Thrombin was obtained from Roche (Mannheim, Germany), and convulxin (Cvx) (ligand of glycoprotein VI from the snake venom *Crotalus durissus terrificus*) was purchased from LOXO (Dossenheim, Germany). SB202190 and z-DEVD-fmk were purchased from Calbiochem (Schwalbach, Germany), and ABT-737 was provided by Selleck Chemicals (Munich, Germany). Antibodies against p38, phospho-p38, and actin were obtained from Sigma (Munich, Germany). Antibodies against phospho-HSP27, phospho-cPLA<sub>2</sub>, cPLA<sub>2</sub>, and caspase 3 were from Cell Signaling (Frankfurt, Germany). Anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase were obtained from Amersham, Pharmacia Biotech (Freiburg, Germany). The fluorescent dyes Annexin-V-PE and PAC-1 were obtained from BD-Bioscience (Heidelberg, Germany). JC1 was obtained from Invitrogen (Eugen, Germany), and H<sub>2</sub>DCF-DA was purchased from Molecular Probes (Göttingen, Germany).

**Platelet preparation.** Human platelets were prepared and used as previously reported,<sup>57</sup> with minor modifications. Blood was obtained from healthy volunteers according to our institutional guidelines and the Declaration of Helsinki. Our studies with human platelets were approved and recently (24 September, 2008) reconfirmed by the local ethics committee of the University of Würzburg (Studies Number 67/92 and 114/04).

Blood was collected into 1/7 volume of ACD solution (12 mM citric acid, 15 mM sodium citrate, 25 mM D-glucose, and 2 μM EGTA, final concentrations). Platelet rich plasma was obtained by a 5-min centrifugation at  $330 \times g$ . To reduce leukocyte contamination, Platelet rich plasma was diluted 1 : 1 with PBS and centrifuged at  $240 \times g$  for 10 min. The supernatant was centrifuged for 10 min at  $430 \times g$ , and the pelleted platelets were washed once in CGS buffer (120 mM sodium chloride, 12.9 mM trisodium citrate, and 30 mM D-glucose, pH 6.5) and resuspended in HEPES buffer (150 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 10 mM D-glucose, and 10 mM HEPES, pH 7.4) without stirring; 1 mM CaCl<sub>2</sub> was added directly before platelet stimulation.

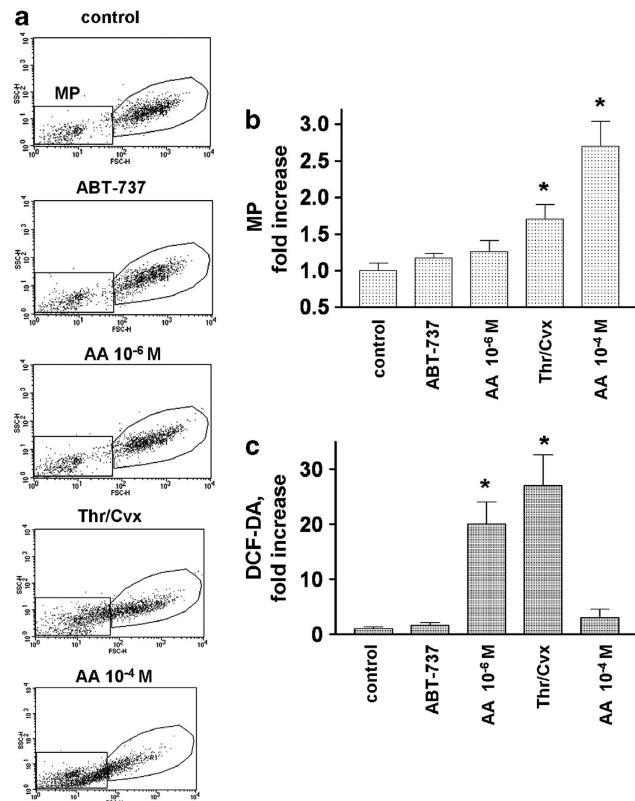
WP were used at a concentration of  $3 \times 10^6$ /ml for western blot analysis and TxS activity, and a concentration of  $1 \times 10^7$ /ml was used for flow cytometry analysis.

**Flow cytometry (FACS) analysis.** FACS analysis was performed using a Becton Dickinson FACS Calibur with CELLQuest software, version 3.1f (Becton Dickinson, Heidelberg, Germany). For the detection of surface PS or activated αIIbβ3 integrins, WP (50 μl) were labeled with Annexin V-PE or PAC-1-FITC (both 1 : 10 dilution) for 10 min at RT after agonist stimulation. The platelets were then diluted 10 times with Annexin V-binding solution (140 mM NaCl, 10 mM HEPES, and 2.5 mM CaCl<sub>2</sub>) for Annexin V or PBS for PAC-1 and immediately analyzed by flow cytometry.

**Analysis of mitochondrial membrane potential.** The ΔΨ<sub>m</sub> in human platelets was analyzed with JC1 dye (1.5 μM final concentration). JC1 was incubated with WP (50 μl) for 10 min at RT, and the samples were diluted (1 : 10) in PBS and analyzed by FACS. Green fluorescence (FL1) and red fluorescence (FL2) were measured in logarithmic scales using voltage settings of 710 and 588, respectively. Compensation settings (FL1-%FL2 and FL2-%FL1) were performed using FITC and PE beads (BD-Bioscience). JC1 is sensitive to ΔΨ<sub>m</sub>, and the ratio of fluorescence in FL2 to FL1 corresponds to changes in ΔΨ<sub>m</sub>.

**Measurement of ROS production.** Intracellular ROS production was analyzed by H<sub>2</sub>DCF-DA, as described previously.<sup>58</sup> Briefly, WP were preloaded with 10 μM H<sub>2</sub>DCF-DA for 30 min at 37 °C in HEPES buffer. H<sub>2</sub>DCF-DA is a cell-permeable, nonfluorescent dye converted intracellularly to cell-impermeable H<sub>2</sub>DCF by intracellular esterases; the dye can then be oxidized by ROS to the fluorescent species (DCF) that is measured by flow cytometry. After incubation with agonists, the reaction was stopped by diluting the platelets in PBS for FACS analysis.

**Measurement of thromboxane synthase activity.** The TxS activity (TxA<sub>2</sub> synthase) was measured as described<sup>59</sup> using a method based on



**Figure 6** Microparticle generation and ROS production from platelets activated by different stimulators. Human WP were stimulated with ABT-737 (1 μM, 60 min), Thr/Cvx (5 mU/5 ng/ml, 2 min), and AA at the indicated concentrations (5 min) and processed for FACS analysis of MP formation, as assessed by the SSC/FSC plot (a and b) and (c) ROS production (DCF-DA fluorescence). The MP counts and DCF-DA fluorescence are presented as a fold increase compared with the control taken as 1. Data are presented as means ± S.E.M, n = 4; \*P < 0.05 compared with the control. (a) Representative plots (SSC/FSC) of the original data

malondialdehyde formation by TxA<sub>2</sub> synthase. For the experiments, 0.2-ml aliquots of WP ( $3 \times 10^9$ /ml) were incubated with the reagents at 37 °C with gentle shaking; the reaction was stopped by the addition of 0.115 ml of ice-cold trichloroacetic acid (20% wt/vol). The samples were kept on ice for 10 min and then centrifuged for 10 min at 14 000 r.p.m. Equal volumes of thiobarbituric acid (0.53% wt/vol) and supernatant were combined and incubated for 30 min at 70 °C. The mixture was then incubated for 30 min at room temperature, and fluorescence was measured with a Wallac Victor2 1420 Multilabel counter (Perkin Elmer Wallac Life and Analytical Sciences, Boston, MA, USA) at excitation and emission wavelengths of 520 nm and 550 nm, respectively. Standards were prepared from the stable malondialdehyde derivative tetraethoxypropane with thiobarbituric acid in the presence of trichloroacetic acid.

**Western blot analysis.** For western blot analysis, WP were added directly to sodium dodecyl sulfate (SDS) gel-loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), as described.<sup>57</sup> The separated proteins were transferred to a nitrocellulose membrane and incubated with the primary antibody overnight at 4 °C. For visualization of the signal, goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody, followed by ECL detection (Amersham, Pharmacia Biotech).

**Data analysis.** All experiments were performed with at least  $n = 4$ , and the combined data are expressed as means  $\pm$  S.E.M. Differences between groups were analyzed by ANOVA, followed by Bonferroni's test; Student's *t*-test was used when appropriate. A value of  $P < 0.05$  was considered statistically significant.

### Conflict of Interest

The authors declare no conflict of interest.

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