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ORIGINAL ARTICLE

N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine induces apoptosis through the activation of caspases-3 and -8 in human platelets. A role for endoplasmic reticulum stress

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Summary. *Background:* Apoptosis or programmed cell death involves a number of biochemical events, including the activation of caspases, which lead to specific cell morphology changes and ultimately cell death. Traditionally, two apoptotic pathways have been described: the cell-surface death receptor-dependent extrinsic pathway and the mitochondria-dependent intrinsic pathway. Alternatively, apoptosis has been reported to be induced by endoplasmic reticulum (ER) stress, which is mainly induced by a reduction in intraluminal free Ca^{2+} concentration ($[Ca^{2+}]_{ER}$). *Objectives:* The present study aimed to investigate the development of apoptotic events after ER stress induced by N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), an ER Ca^{2+} chelator, in human platelets. *Methods:* Changes in cytosolic free Ca^{2+} concentration, caspase activity and phosphatidylserine externalization were determined by fluorimetric techniques. *Results:* Our results indicate that TPEN reduces the amount of free Ca^{2+} releasable by the Ca^{2+}-mobilizing agonist thrombin. TPEN induced activation of caspase-3, -8 and -9 and subsequent phosphatidylserine externalization. The ability of TPEN to induce phosphatidylserine externalization was smaller than that of thrombin. In addition, TPEN was able to induce phosphorylation of the eukaryotic initiation factor 2α (eIF2α). TPEN-mediated caspase-3 activation requires functional caspase-8, but is independent of H_2O_2 generation. Activation of caspase-3 and -8 by TPEN was prevented by salubrinal, an agent that prevents ER stress-induced apoptosis. *Conclusion:* These findings provide experimental evidence for the existence of ER stress-mediated apoptosis in human platelets, a process that might limit platelet life span upon prolonged stimulation with agonists.

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Introduction

The physiological role of the endoplasmic reticulum (ER) includes the synthesis, folding and modification of secretory and transmembrane proteins, and regulation of cellular Ca^{2+} homeostasis [1]. ER function requires a high level of Ca^{2+}, an oxidative environment and a number of resident proteins, called chaperons, which regulate protein folding. The ER has a high sensitivity to alterations in Ca^{2+} homeostasis and low luminal concentrations of Ca^{2+} ($[Ca^{2+}]_{ER}$) are able to produce oxidative stress and accumulation of unfolded and misfolded proteins, resulting in ER dysfunction and stress [1,2], which initiates the development of apoptosis. There are three known apoptotic pathways triggered by ER stress: the CHOP(C/EBP homologous protein)/GADD153 pathway, the cJUN NH2-terminal kinase (JNK) pathway and the caspase pathway [1].

Caspases are cysteine aspartate proteases involved in apoptosis, leading to the loss of cellular structure and function, phosphatidylserine exposure and cell death [3]. In particular, caspase-3, -8 and -9 play a relevant role in apoptosis: caspase-9 is activated by mitochondrial cytochrome *c* release, caspase-8 activation is induced by the occupation of the Fas/CD95 receptor, and caspase-3 is an executioner caspase activated by multiple pathways [4]. Recent studies have demonstrated that ER stress induces activation of caspase-2, -8, -9, -11 and -12 [5–8].

Platelets express several components of the apoptotic machinery. Platelet activation by thrombin, a Ca^{2+} mobilizing agonist, induces H_2O_2-mediated apoptotic events, including cytochrome *c* release, caspase-3 and -9 activation, phosphatidylserine exposure and Bid, Bax and Bak activation [9–13], although it is unknown whether depletion of the intracellular Ca^{2+} stores, especially the dense tubular system, the analogue of the ER in platelets, has a role in the development of apoptotic events. Here we have investigated the development of apoptotic events after ER stress in human platelets induced by

N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a cell-permeant ER Ca^{2+} chelator that induces ER stress [14].

Materials and methods

Materials

Fura-2 AM and calcein-AM were from Molecular Probes (Leiden, the Netherlands). Apyrase (grade VII), aspirin, thrombin, thapsigargin (TG), CHAPS, 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ), catalase, fluorescein isothiocyanate-labeled annexin V,N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), glutaraldehyde, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-amc), N-acetyl-Leu-Glu-His-Asp-7-amido-4-methylcoumarin (AC-LEHD-amc), anti-actin antibody and BSA were from Sigma (Poole, Dorset, UK). The caspase inhibitors z-IETD-fmk and z-LEHD-fmk were from BD biosciences (Madrid, Spain). The caspase-8 substrate N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (AC-IETD-amc) was from Bachem (Weil am Rhein, Germany). Salubrinal was from Tocris (Bristol, UK). Rabbit anti-phospho-eIF2α (Ser^{51}) antibody and anti-caspase-3 antibody was from Cell Signalling (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody and anti-caspase-8 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-mouse IgG antibody was from Amersham, (Buckinghamshire, UK). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, UK). All other reagents were of analytical grade.

Platelet preparation

Blood was collected from healthy volunteers in accordance with the Declaration of Helsinki and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in millimolar): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was prepared by centrifugation for 5 min at $700 \times g$ and aspirin (100 μM) and apyrase (40 μg mL^{-1}) were added [15]. Cells were collected by centrifugation at $350 \times g$ for 20 min and resuspended in HEPES-buffered saline (HBS) containing (in millimolar): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45 and supplemented with 0.1% w/v BSA and 40 μg mL^{-1} apyrase.

Platelet viability

Cell viability was assessed by calcein loading of the cells and trypan blue exclusion technique. For calcein loading, platelets were incubated for 30 min with 5 μM calcein-AM at 37 °C, centrifuged and suspended in HBS. Cells were treated with the agents used, centrifuged and resuspended in HBS. Two-millilitre aliquots were used for fluorescence recording (spectrophotometer, Varian Ltd, Madrid, Spain) at λ ex: 494 nm and λ em: 535 nm. In this assay ~95% of cells were viable in our platelet suspensions, a result further confirmed by the trypan blue exclusion technique.

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_c$)

Cells were incubated at 37 °C with 2 μM fura-2 AM for 45 min and resuspended in HBS. Fluorescence was recorded from 1 mL aliquots of magnetically stirred platelet suspensions (10^8 cells mL^{-1}) at 37 °C using the fluorescence Spectrophotometer (Varian Ltd) with λ ex: 340 and 380 nm and λ em: 505 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz *et al*. Ca^{2+} release and influx was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after addition of $CaCl_2$ [16].

Determination of phosphatidylserine externalization

Phosphatidylserine exposure was determined according to a published procedure [12]. Briefly, samples of cell suspensions (500 μL) were transferred to 500 μL ice-cold 1% (w/v) glutaraldehyde in phosphate-buffered saline (PBS) for 10 min. Cells were then incubated for 10 min with annexin V-FITC (0.6 μg mL^{-1}) in PBS supplemented with 0.5% (w/v) BSA. Cells were collected by centrifugation for 60 s at $3000 \times g$ and resuspended in PBS. Cell staining was measured using a fluorescence Spectrophotometer with λ ex: 496 nm and λ em: 516 nm. Phosphatidylserine externalization was estimated numerically as arbitrary units of mean fluorescence intensity and presented as fold increases over control.

Western blotting

Proteins were separated by 10% SDS–PAGE and electrophoretically transferred, for 2 h at 0.8 mA cm^{-2}, in a semi-dry blotter (Hoefer Scientific, Newcastle, Staffs, 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 anti-phospho-eIF2α, anti-caspase-3, anti-caspase-8 or anti-actin antibodies diluted 1:1000 in TBST for 2 h. To detect the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated anti-IgG 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.

Caspase activity assay

To determinate caspase-3, -8 and -9 activity platelet lysates were incubated with 2 mL of substrate solution (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 8.25 μM of caspase substrate) for 2 h at 37 °C as described [13]. Substrate cleaving was measured with a fluorescence

spectrophotometer with λ ex: 360 nm and λ em: 460 nm. Preliminary experiments reported that caspase-3, -8 and -9 substrate cleaving was not detected in the presence of their respective inhibitors. The activity of caspase-3, -8 and -9 was calculated from the cleavage of the respective specific fluorogenic substrate AC-DEVD-amc, AC-IETD-amc and AC-LEHD-amc, respectively, following the instructions of the manufacturer.

Statistical analysis

Data are shown as mean ± SEM. Analysis of statistical significance was performed using Student's *t*-test. $P < 0.05$ was considered to be significant for a difference.

Results

TPEN reduces thrombin-evoked Ca^{2+} mobilization

In a Ca^{2+}-free medium, treatment of fura-2-loaded platelets with thrombin (1 U mL^{-1}) evoked a transient elevation in $[Ca^{2+}]_c$ due to Ca^{2+} release from internal stores. Subsequent addition of 300 μM Ca^{2+} to the external medium induced a sustained increase in $[Ca^{2+}]_c$ indicative of Ca^{2+} entry (Fig. 1A). TPEN has been presented as a membrane-permeant multivalent cation chelator, with moderate affinity for Ca^{2+} (Kd ~130 μM), used to rapidly and reversibly chelate Ca^{2+} within the intracellular stores [17]. As shown in Fig. 1(A), TPEN (200 μM) did not modify $[Ca^{2+}]_c$ in a Ca^{2+}-free medium; however, due to *in situ* Ca^{2+} chelation in the ER, in the presence of TPEN, thrombin-evoked Ca^{2+} release was significantly reduced ($P < 0.05$). The chelating effects of TPEN were achieved at times as short as 1 min after its addition and were not enhanced by further incubation times (Fig. 1B). In the presence of TPEN, thrombin-evoked Ca^{2+} entry was significantly attenuated, possibly as a result of intracellular Ca^{2+} chelation and competition with the Ca^{2+} indicator (Fig. 1B; $P < 0.05$).

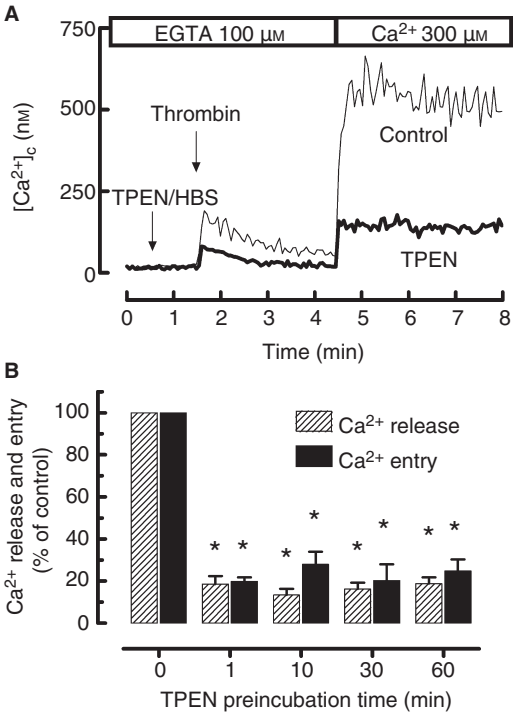

Fig. 1. TPEN reduces thrombin-evoked Ca^{2+} mobilization in human platelets. (A) Fura-2-loaded human platelets were suspended in a Ca^{2+}-free medium (100 μM EGTA was added) and then were treated with 200 μM TPEN or HBS (as control). Cells were then stimulated with 1 U/mL^{-1} thrombin and 3 min later $CaCl_2$ (final concentration 300 μM) was added to initiate Ca^{2+} entry. Traces are representative of six independent experiments. (B) Histograms represent thrombin-induced Ca^{2+} release and entry, as indicated, in cells preincubated in the presence of TPEN for various periods of time (1–60 min) or in its absence ($t = 0$). *$P < 0.05$ compared with controls (cells not treated with TPEN).

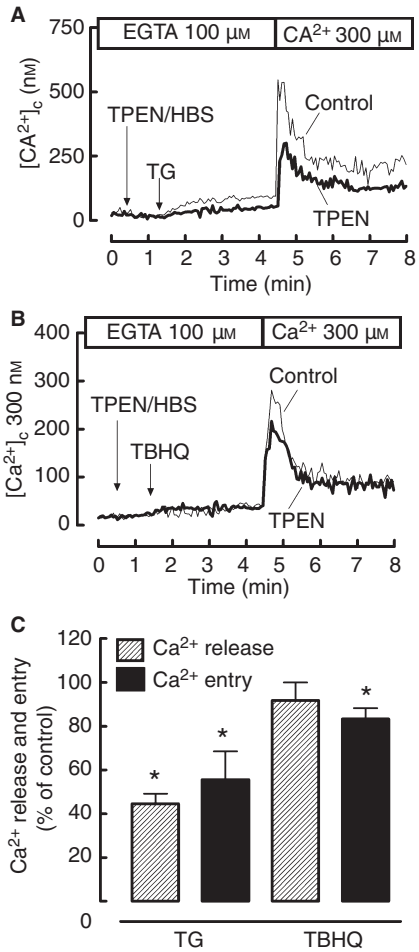

Fig. 2. Effect of TPEN on TG- and TBHQ-evoked Ca^{2+} mobilization in human platelets. (A and B) Fura-2-loaded human platelets were suspended in a Ca^{2+}-free medium and then were treated with 200 μM TPEN or the vehicle (as control). Cells were then stimulated with 10 nM TG (A) or 20 μM TBHQ (B) and 3 min later $CaCl_2$ (final concentration 300 μM) was added to initiate Ca^{2+} entry. Traces are representative of six independent experiments. (C) Histograms represent TG- or TBHQ-induced Ca^{2+} release and entry, as indicated, in cells preincubated for 1 min with TPEN. *$P < 0.05$ compared with controls (cells not treated with TPEN).

We have previously reported that thrombin releases Ca^{2+} from two intracellular stores in human platelets, the dense tubular system (DTS) and the lysosome-related (acidic) organelles [16,18]. Two different isoforms of sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPases (SERCA) have been identified in these stores [19–21]; SERCA 2b, insensitive to TBHQ but inhibited by low concentrations of TG, is located in the DTS [20,22], while SERCA 3, with low sensitivity to TG [23,24] and sensitive to TBHQ [22], is located in the acidic stores [16,18]. We have now investigated whether TPEN affects Ca^{2+} mobilization from both intracellular stores. In a Ca^{2+}-free medium, treatment with 10 nM TG or 20 μM TBHQ resulted in small and sustained increases in $[Ca^{2+}]_c$ due to Ca^{2+} efflux from the DTS or the acidic stores, respectively. Subsequent addition of Ca^{2+} induced a sustained increase in $[Ca^{2+}]_c$ indicative of store-operated Ca^{2+} entry (Fig 2A and B). Preincubation with TPEN for 1 min significantly reduced TG-evoked Ca^{2+} release (Fig. 2A and C; $P < 0.05$) but had a negligible effect on TBHQ-induced response (Fig. 2B and C). These findings suggest that TPEN is an efficient chelator of Ca^{2+} accumulated in the DTS but had minor effects on the acidic organelles.

TPEN induces phosphatidylserine externalization, phosphorylation of eIF2α and caspase activation

Phosphatidylserine exposure has been presented as a major apoptotic feature in human platelets [9,12]. Our results indicate that 200 μM TPEN induced a time-dependent phosphatidylserine exposure, which was detectable at times as short as 1 min with a 1.20 ± 0.10-fold increase, and reached a maximal effect after 60 min of treatment with a 2.14 ± 0.25-fold increase (Fig. 3A). The effect of TPEN on phosphatidylserine externalization was significantly smaller than that induced by thrombin (Fig. 3A).

The effect of TPEN on ER stress was tested by analyzing the phosphorylation of eIF2α at Ser^{51}, a crucial cellular response to ER stress [25]. Phosphorylation of eIF2α was detected by Western blotting using a specific anti-phospho-eIF2α (Ser^{51}) antibody [26]. As shown in Fig. 3(B), TPEN induced a time-

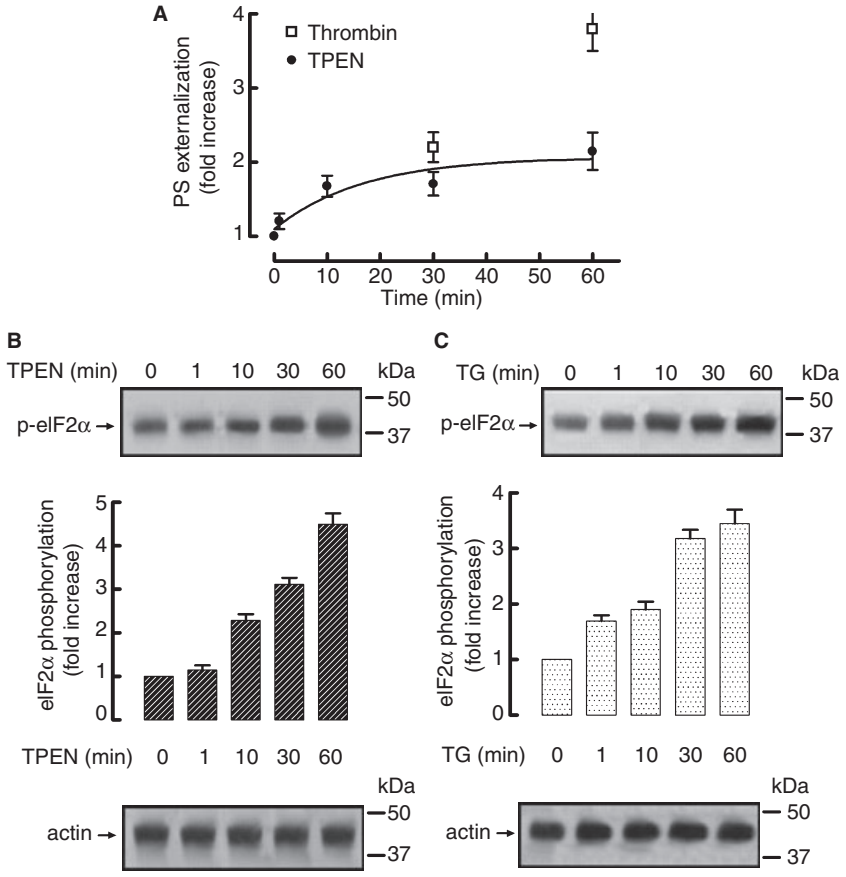

Fig. 3. TPEN induces phosphatidylserine exposure and phosphorylation of eIF2α in human platelets. (A) Platelets were treated in a medium containing 1 mM $CaCl_2$ with 200 μM TPEN for various periods of time (1–60 min) or with 1 U mL^{-1} thrombin for 30 and 60 min, as indicated, and PS exposure was determined. (B and C) Platelets were treated in a medium containing 1 mM $CaCl_2$ with 200 μM TPEN (B) or 1 μM TG (C) for various periods of time (1–60 min), as indicated, and phosphorylation of eIF2α was determined by Western blotting. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Values are presented as means ± SEM of six separate experiments and expressed as fold-increase over the pretreatment level (experimental/control).

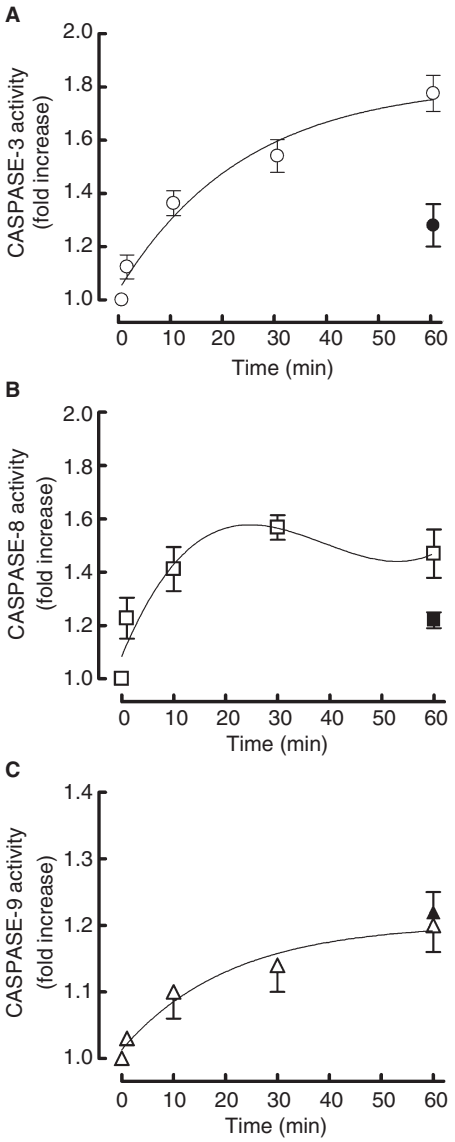

Fig. 4. TPEN-induces activation of caspase-3, -8 and -9 in platelets. Platelets were treated in a Ca^{2+}-free medium with 200 μM TPEN for various periods of time (1–60 min) or with 1 μM TG for 60 min and caspase-3 (A), -8 (B) and -9 (C) activities were estimated. Values are presented as means ± SEM of six separate experiments and expressed as fold increase over the pretreatment level (experimental/control).

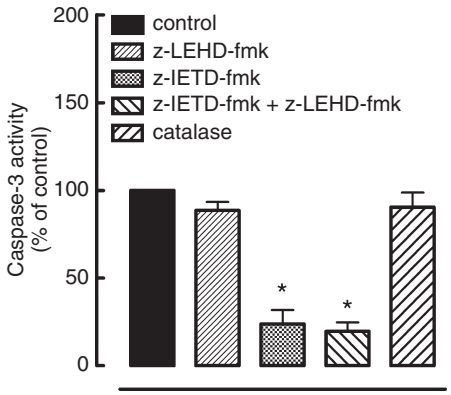

Fig. 5. Role of caspase-8 and -9 and hydrogen peroxide in TPEN-evoked caspase-3 activation. Human platelets were preincubated at 37 °C in the absence or presence of 40 μM z-LEHD-fmk (caspase-9 inhibitor), 100 μM z-IETD-fmk (caspase-8 inhibitor) or both for 90 min or with 300 U mL^{-1} catalase for 10 min. Platelets were treated in a Ca^{2+}-free medium (100 μM EGTA was added) with 200 μM TPEN for 30 min and caspase-3 activity was estimated. Values are presented as means ± SEM of six separate experiments and expressed as percentage of control (TPEN-treated platelets in the absence of inhibitors). *$P < 0.05$ compared with controls.

dependent phosphorylation of eIF2α, reaching a maximal effect after 60 min of treatment with a 4.5 ± 0.2-fold increase. Similar results were observed with 1 μM TG, which reduces $[Ca^{2+}]_{ER}$ by inhibition of SERCA (Fig. 3C).

Treatment of platelets with 200 μM TPEN for various periods of time (1–60 min) induced a time-dependent activation of caspase-3. TPEN induced a detectable increase in caspase-3 activity after 1 min of treatment with a 1.12 ± 0.04-fold increase (Fig. 4A; $P < 0.05$). Although maximal caspase-3 activation was not reached in the range of time studied, after 60 min stimulation with TPEN caspase-3 activity reached a 1.77 ± 0.06-fold increase (Fig. 4A). TPEN also induced a time-dependent activation of caspase-8, reaching a maximum after 30 min stimulation with a 1.56 ± 0.04-fold increase (Fig. 4B), and caspase-9, although the extent of activation was smaller than that observed for caspase-3 or -8 (Fig. 4C). Caspase activation was also found after platelet stimulation with TG (1 μM), although, except for caspase-9, the effect of TG was smaller than that of TPEN (Fig. 4).

We have now found that TPEN activates caspase-3 to a similar extent as 10 μM H_2O_2, as previously reported [12], which we have previously found to be generated by physiological concentrations of agonists [27]. Hence, we have investigated whether the effect of TPEN was mediated by endogenous H_2O_2 generation using catalase, an enzyme that activates H_2O_2 decomposition. Treatment of platelets for 10 min with 300 U mL^{-1} catalase did not significantly modify TPEN-mediated caspase-3 activation (caspase-3 activity was 90.3 ± 8.5% of control in the presence of catalase; Fig. 5), thus suggesting that caspase-3 activation by TPEN is not mediated by generation of H_2O_2.

We have further investigated whether TPEN-mediated caspase-3 activation requires caspase-9, by using z-LEHD-fmk, a specific caspase-9 inhibitor [28], or caspase-8 activity, by using z-IETD-fmk, a caspase-8 inhibitor [29]. As shown in Fig. 5, treatment for 90 min with 40 μM z-LEHD-fmk had a minor effect on TPEN-mediated caspase-3 activation. In contrast, treatment for 90 min with the caspase-8 inhibitor z-IETD-fmk significantly inhibited TPEN-mediated caspase-3 activation by 77% (Fig. 5; $P < 0.05$). Incubation of platelets with a combination of z-LEHD-fmk and z-IETD-fmk induced similar effects than z-IETD-fmk alone, further suggesting that caspase-9 has a minor effect on the activation of caspase-3 by TPEN.

Finally, we have investigated whether caspase activation by TPEN is mediated by ER stress by using salubrinal, a cell-permeant and selective inhibitor of the phosphatase complexes

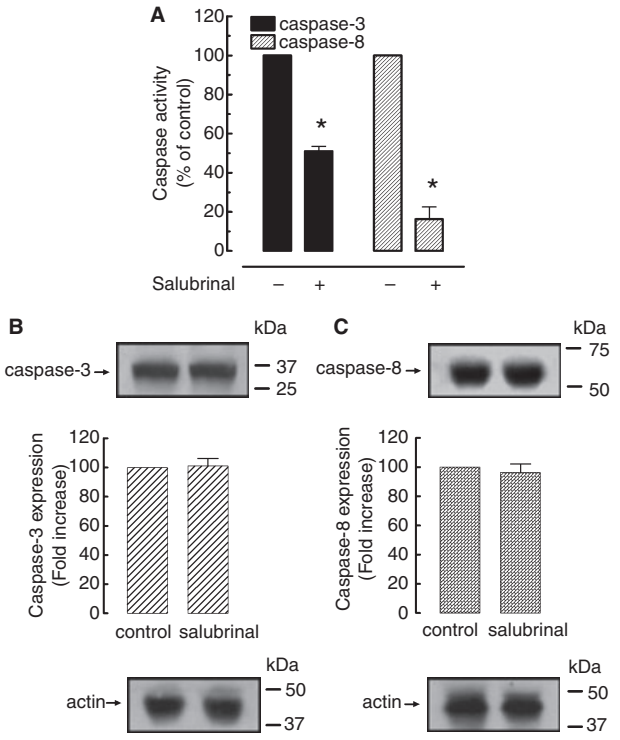

Fig. 6. Salubrinal attenuates TPEN-induced activation of caspase-3 and -8 in platelets. (A) Platelets were preincubated for 30 min with 30 μM salubrinal and then treated in a Ca^{2+}-free medium with 200 μM TPEN for a further 30 min. Caspase activity was determined as described in 'Material and methods'. (B and C) Platelets were treated with salubrinal and caspase-3 (B) or caspase-8 (C) expression was determined by Western blotting. Samples were reprobed with anti-actin antibody for protein loading control. Immunoblot panels show results from one experiment representative of five others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Values are presented as means ± SEM of six separate experiments and expressed as percentage of control (platelets not treated with salubrinal).

that dephosphorylate eIF2α, which protects against ER stress-induced apoptosis [30]. Treatment of human platelets for 30 min with 30 μM salubrinal significantly attenuated TPEN-evoked activation of caspase-3 and -8 by 50% and 84%, respectively (Fig. 6A; $P < 0.05$). This effect cannot be attributed to a reduction in caspase expression as shown in Fig. 6(B and C).

Discussion

Platelets have been reported to express several components of the apoptotic machinery, including proapoptotic proteins, such as Bax, Bak and Bid [9,13] and caspase-3 and -9 [31,32]. Platelets also express caspase-8 and -10 [33]; however, caspase-12 has not been found in platelets, despite it being highly expressed in mature megakaryocytes [34]. Apoptosis in platelets has been described under certain storage conditions and after stimulation with agonists, a process mostly associated with activation of the intrinsic apoptotic pathway [9,12,13], and is involved in the determination of platelet life span [35]. Here we show for the first time activation of apoptotic events upon ER stress induced by the cell-permeant Ca^{2+} chelator TPEN in human platelets. TPEN attenuates $[Ca^{2+}]_{ER}$ available to agonists, such as thrombin, thus explaining the reduced thrombin-evoked Ca^{2+} release observed in the presence of TPEN. TPEN (200 μM) was unable to completely inhibit thrombin-stimulated Ca^{2+} release even after 60 min of treatment. Hence, we tested the effect of TPEN on Ca^{2+} efflux from the DTS or the acidic organelles in platelets, whose Ca^{2+} gradients are maintained by SERCA2b and 3, respectively [19–22,36]. It is known that SERCA2b and 3 are selectively inhibited by either low concentrations of TG or TBHQ, respectively [17,19,37]. Our results indicate that Ca^{2+} release from the DTS was reduced by TPEN, which had no effect on Ca^{2+} release from the acidic stores. Although speculative, we believe that the effect of TPEN is unlikely to be mediated through a reduction of the Ca^{2+} leakage rate through the membranes of the stores because this action would be expected to affect both Ca^{2+} compartments, which is not the case. Therefore, our results are more likely to be attributed to a selective effect of TPEN on Ca^{2+} accumulated in the DTS, the analogous of the ER in human platelets.

We have found that TPEN was able to induce the development of apoptotic events, such as phosphatidylserine externalization, in human platelets, in agreement with previous studies in other cell types [38]. Our study demonstrates that TPEN induces time-dependent activation of caspase-3, which paralleled phosphatidylserine exposure and requires the activity of caspase-8. In contrast to the intrinsic apoptotic pathway in platelets, ER stress-mediated caspase-3 activation was not dependent on the generation of H_2O_2 as demonstrated by the use of catalase. Caspase activation was also achieved by TG, an agent that induces ER stress by depletion of the Ca^{2+} stores [39].

Phosphorylation of eIF2α at Ser^{51} is an important cellular response to ER stress that represses general protein synthesis [26]. We have found that TPEN, as well as the SERCA inhibitor TG, is able to induce a time-dependent increase in the phosphorylation of eIF2α at Ser^{51}, thus supporting the ability of TPEN to induce ER stress in human platelets.

ER stress has also been suggested to be associated with the activation of other intrinsic pathways of apoptosis. Activation of caspase-8 has been reported to induce cleaving of the BH3-only protein Bid [40], which acts as a signal on the outer mitochondrial membrane, thus promoting oligomerization of the proapoptotic proteins Bax and Bak and cytochrome *c* release [41]. This pathway might explain the activation of caspase-9 by TPEN in human platelets. Several apoptotic pathways, including those activated by ER stress, are also involved in the maturation of hematopoietic cells and particularly in platelet formation [42,43], where the expression of Ca^{2+}-ATPases, especially SERCA3, has been reported to play an important role [44,45].

In summary, our results demonstrate for the first time that a reduction in available $[Ca^{2+}]_{ER}$, which leads to ER stress, as demonstrated by phosphorylation of eIF2α at Ser^{51}, results in

time-dependent activation of caspase-3, a process that requires caspase-8 activity, and phosphatidylserine externalization, two features that are characteristic of the development of apoptosis in human platelets. Although we cannot rule out the possibility that chelation of heavy metals by TPEN, especially Zn^{2+}, participates in TPEN-mediated apoptosis in platelets, the inhibitory role of salubrinal on TPEN-induced response and the effect of TPEN on eIF2α phosphorylation, a mechanism of the unfolded protein response used by cells to cope with ER stress [46], support that ER stress is involved in TPEN-induced caspase activation. These findings provide evidence for the existence of a new apoptotic pathway in platelets that might limit platelet life span upon prolonged stimulation with physiological agonists.

Acknowledgements

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Groenendyk J, Michalak M. Endoplasmic reticulum quality control and apoptosis. *Acta Biochim Pol* 2005; **52**: 381–95.
2 Rao RV, Ellerby HM, Bredesen DE. Coupling endoplasmic reticulum stress to the cell death program. *Cell Death Differ* 2004; **11**: 372–80.
3 Stennicke HR, Jurgensmeier JM, Shin H, Deveraux Q, Wolf BB, Yang X, Zhou Q, Ellerby HM, Ellerby LM, Bredesen D, Green DR, Reed JC, Froelich CJ, Salvesen GS. Pro-caspase-3 is a major physiologic target of caspase-8. *J Biol Chem* 1998; **273**: 27084–90.
4 Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* 2002; **9**: 459–70.
5 Jimbo A, Fujita E, Kouroku Y, Ohnishi J, Inohara N, Kuida K, Sakamaki K, Yonehara S, Momoi T. ER stress induces caspase-8 activation, stimulating cytochrome c release and caspase-9 activation. *Exp Cell Res* 2003; **283**: 156–66.
6 Cheung HH, Lynn Kelly N, Liston P, Korneluk RG. Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. *Exp Cell Res* 2006; **312**: 2347–57.
7 Endo M, Mori M, Akira S, Gotoh T. C/EBP homologous protein (CHOP) is crucial for the induction of caspase-11 and the pathogenesis of lipopolysaccharide-induced inflammation. *J Immunol* 2006; **176**: 6245–53.
8 Shiraishi H, Okamoto H, Yoshimura A, Yoshida H. ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1. *J Cell Sci* 2006; **119**: 3958–66.
9 Leytin V, Allen DJ, Mykhaylov S, Lyubimov E, Freedman J. Thrombin-triggered platelet apoptosis. *J Thromb Haemost* 2006; **4**: 2656–63.
10 Rosado JA, Lopez JJ, Gomez-Arteta E, Redondo PC, Salido GM, Pariente JA. Early caspase-3 activation independent of apoptosis is required for cellular function. *J Cell Physiol* 2006; **209**: 142–52.
11 Leytin V, Allen DJ, Lyubimov E, Freedman J. Higher thrombin concentrations are required to induce platelet apoptosis than to induce platelet activation. *Br J Haematol* 2007; **136**: 762–4.
12 Lopez JJ, Salido GM, Gomez-Arteta E, Rosado JA, Pariente JA. Thrombin induces apoptotic events through the generation of reactive oxygen species in human platelets. *J Thromb Haemost* 2007; **5**: 1283–91.
13 Lopez JJ, Salido GM, Pariente JA, Rosado JA. Thrombin induces activation and translocation of Bid, Bax and Bak to the mitochondria in human platelets. *J Thromb Haemost* 2008; **6**: 1780–8.
14 Chimienti F, Seve M, Richard S, Mathieu J, Favier A. Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors. *Biochem Pharmacol* 2001; **62**: 51–62.
15 Redondo PC, Jardin I, Hernandez-Cruz JM, Pariente JA, Salido GM, Rosado JA. Hydrogen peroxide and peroxynitrite enhance Ca^{2+} mobilization and aggregation in platelets from type 2 diabetic patients. *Biochem Biophys Res Commun* 2005; **333**: 794–802.
16 Lopez JJ, Redondo PC, Salido GM, Pariente JA, Rosado JA. Two distinct Ca^{2+} compartments show differential sensitivity to thrombin, ADP and vasopressin in human platelets. *Cell Signal* 2006; **18**: 373–81.
17 Hofer AM, Fasolato C, Pozzan T. Capacitative Ca2+ entry is closely linked to the filling state of internal Ca^{2+} stores: a study using simultaneous measurements of ICRAC and intraluminal [Ca^{2+}]. *J Cell Biol* 1998; **140**: 325–34.
18 Lopez JJ, Camello-Almaraz C, Pariente JA, Salido GM, Rosado JA. Ca^{2+} accumulation into acidic organelles mediated by Ca^{2+}-and vacuolar H^+-ATPases in human platelets. *Biochem J* 2005; **390**: 243–52.
19 Papp B, Enyedi A, Kovacs T, Sarkadi B, Wuytack F, Thastrup O, Gardos G, Bredoux R, Levy-Toledano S, Enouf J. Demonstration of two forms of calcium pumps by thapsigargin inhibition and radioimmunoblotting in platelet membrane vesicles. *J Biol Chem* 1991; **266**: 14593–6.
20 Cavallini L, Coassin M, Alexandre A. Two classes of agonist-sensitive Ca^{2+} stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin. *Biochem J* 1995; **310**: 449–52.
21 Kovacs T, Berger G, Corvazier E, Paszty K, Brown A, Bobe R, Papp B, Wuytack F, Cramer EM, Enouf J. Immunolocalization of the multisarco/endoplasmic reticulum Ca2+ ATPase system in human platelets. *Br J Haematol* 1997; **97**: 192–203.
22 Papp B, Enyedi A, Paszty K, Kovacs T, Sarkadi B, Gardos G, Magnier C, Wuytack F, Enouf J. Simultaneous presence of two distinct endoplasmic-reticulum-type calcium-pump isoforms in human cells. Characterization by radio-immunoblotting and inhibition by 2,5-di-(t-butyl)-1,4-benzohydroquinone. *Biochem J* 1992; **288**: 297–302.
23 Bobe R, Bredoux R, Wuytack F, Quarck R, Kovacs T, Papp B, Corvazier E, Magnier C, Enouf J. The rat platelet 97-kDa Ca2+ATPase isoform is the sarcoendoplasmic reticulum Ca2+ATPase 3 protein. *J Biol Chem* 1994; **269**: 1417–24.
24 Wuytack F, Papp B, Verboomen H, Raeymaekers L, Dode L, Bobe R, Enouf J, Bokkala S, Authi KS, Casteels R. A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells. *J Biol Chem* 1994; **269**: 1410–6.
25 Lee YY, Cevallos RC, Jan E. An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2alpha phosphorylation. *J Biol Chem* 2009; **284**: 6661–73.
26 Lewerenz J, Maher P. Basal levels of eIF2alpha phosphorylation determine cellular antioxidant status by regulating ATF4 and xCT expression. *J Biol Chem* 2009; **284**: 1106–15.
27 Rosado JA, Redondo PC, Salido GM, Gomez-Arteta E, Sage SO, Pariente JA. Hydrogen peroxide generation induces pp60src activation in human platelets: evidence for the involvement of this pathway in store-mediated calcium entry. *J Biol Chem* 2004; **279**: 1665–75.
28 Ozoren N, Kim K, Burns TF, Dicker DT, Moscioni AD, El-Deiry WS. The caspase 9 inhibitor Z-LEHD-FMK protects human liver cells while permitting death of cancer cells exposed to tumor necrosis

factor-related apoptosis-inducing ligand. *Cancer Res* 2000; **60**: 6259–65.
29 Bortner CD, Cidlowski JA. Caspase independent/dependent regulation of K^+, cell shrinkage, and mitochondrial membrane potential during lymphocyte apoptosis. *J Biol Chem* 1999; **274**: 21953–62.
30 Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, Yuan J. A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science* 2005; **307**: 935–9.
31 Leytin V, Mykhaylov S, Starkey AF, Allen DJ, Lau H, Ni H, Semple JW, Lazarus AH, Freedman J. Intravenous immunoglobulin inhibits anti-glycoprotein IIb-induced platelet apoptosis in a murine model of immune thrombocytopenia. *Br J Haematol* 2006; **133**: 78–82.
32 Ben Amor N, Pariente JA, Salido GM, Bartegi A, Rosado JA. Caspases 3 and 9 are translocated to the cytoskeleton and activated by thrombin in human platelets. Evidence for the involvement of PKC and the actin filament polymerization. *Cell Signal* 2006; **18**: 1252–61.
33 Piguet PF, Kan CD, Vesin C. Thrombocytopenia in an animal model of malaria is associated with an increased caspase-mediated death of thrombocytes. *Apoptosis* 2002; **7**: 91–8.
34 Kerrigan SW, Gaur M, Murphy RP, Shattil SJ, Leavitt AD. Caspase-12: a developmental link between G-protein-coupled receptors and integrin alphaIIbbeta3 activation. *Blood* 2004; **104**: 1327–34.
35 Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S, Kelly PN, Ekert PG, Metcalf D, Roberts AW, Huang DC, Kile BT. Programmed anuclear cell death delimits platelet life span. *Cell* 2007; **128**: 1173–86.
36 Lopez JJ, Jardin I, Bobe R, Pariente JA, Enouf J, Salido GM, Rosado JA. STIM1 regulates acidic Ca2+ store refilling by interaction with SERCA3 in human platelets. *Biochem Pharmacol* 2008; **75**: 2157–64.
37 Rosado JA, Lopez JJ, Harper AG, Harper MT, Redondo PC, Pariente JA, Sage SO, Salido GM. Two pathways for store-mediated calcium entry differentially dependent on the actin cytoskeleton in human platelets. *J Biol Chem* 2004; **279**: 29231–5.
38 Lee JM, Kim YJ, Ra H, Kang SJ, Han S, Koh JY, Kim YH. The involvement of caspase-11 in TPEN-induced apoptosis. *FEBS Lett* 2008; **582**: 1871–6.
39 Yoshida I, Monji A, Tashiro K, Nakamura K, Inoue R, Kanba S. Depletion of intracellular Ca2+ store itself may be a major factor in thapsigargin-induced ER stress and apoptosis in PC12 cells. *Neurochem Int* 2006; **48**: 696–702.
40 Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; **94**: 491–501.
41 Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev* 2001; **15**: 2922–33.
42 De Botton S, Sabri S, Daugas E, Zermati Y, Guidotti JE, Hermine O, Kroemer G, Vainchenker W, Debili N. Platelet formation is the consequence of caspase activation within megakaryocytes. *Blood* 2002; **100**: 1310–7.
43 Zhang K, Wong HN, Song B, Miller CN, Scheuner D, Kaufman RJ. The unfolded protein response sensor IRE1alpha is required at 2 distinct steps in B cell lymphopoiesis. *J Clin Invest* 2005; **115**: 268–81.
44 Lacabaratz-Porret C, Launay S, Corvazier E, Bredoux R, Papp B, Enouf J. Biogenesis of endoplasmic reticulum proteins involved in Ca^{2+} signalling during megakaryocytic differentiation: an *in vitro* study. *Biochem J* 2000; **350**: 723–34.
45 Chaabane C, Corvazier E, Bredoux R, Dally S, Raies A, Villemain A, Dupuy E, Enouf J, Bobe R. Sarco/endoplasmic reticulum Ca^{2+}-ATPase type 3 isoforms (SERCA3b and SERCA3f): distinct roles in cell adhesion and ER stress. *Biochem Biophys Res Commun* 2006; **345**: 1377–85.
46 Liu CY, Kaufman RJ. The unfolded protein response. *J Cell Sci* 2003; **116**: 1861–2.