Crucial Role for Endoplasmic Reticulum Stress During Megakaryocyte Maturation

Jose J. Lopez, Alberta Palazzo, Chiraz Chaabane, Letizia Albarran, Evelyne Polidano, Kristell Lebozec, Saoussen Dally, Paquita Nurden, Jocelyne Enouf, Najet Debili, Régis Bobe

Objective—Apoptotic-like phase is an essential step for the platelet formation from megakaryocytes. How controlled is this signaling pathway remained poorly understood. The aim of this study was to determine whether endoplasmic reticulum (ER) stress—induced apoptosis occurs during thrombopoiesis.

Approach and Results—Investigation of ER stress and maturation markers in different models of human thrombopoiesis (CHRF, DAMI, MEG-01 cell lines, and hematopoietic stem cells: CD34+) as well as in immature pathological platelets clearly indicated that ER stress occurs transiently during thrombopoiesis. Direct ER stress induction by tunicamycin, an inhibitor of N-glycosylation, or by sarco/endoplasmic reticulum Ca²⁺ ATPase type 3b overexpression, which interferes with reticular calcium, leads to some degree of maturation in megakaryocytic cell lines. On the contrary, exposure to salubrinal, a phosphatase inhibitor that prevents eukaryotic translation initiation factor 2α-P dephosphorylation and inhibits ER stress—induced apoptosis, decreased both expression of maturation markers in MEG-01 and CD34⁺ cells as well as numbers of mature megakaryocytes and proplatelet formation in cultured CD34⁺ cells.

Conclusions—Taken as a whole, our research suggests that transient ER stress activation triggers the apoptotic-like phase of the thrombopoiesis process. (Arterioscler Thromb Vasc Biol. 2013;33:2750-2758.)

Key Words: apoptosis ♦ endoplasmic reticulum stress ♦ platelet ♦ thrombopoiesis

Megakaryocytes are responsible for the production of circulating platelets, essential players in hemostasis and thrombosis, through a maturation process named thrombopoiesis. Thrombopoiesis is a complex process involving distinct steps: DNA endoduplication, increase of both ploidy and number of storage organelles, and cytoplasmic fragmentation. The latter results in the formation of proplatelets and platelet shedding and is characterized by deep changes in cytoskeleton and calcium signaling organization. Although several studies support the idea that thrombopoiesis activate intrinsic apoptotic signaling pathway, more recent publications suggest that apoptosis needs in fact to be restrained during thrombopoiesis and question the suggested role of mitochondrial apoptosis in megakaryocyte to generate platelets.

Josefsson et al. showed that deletion of prosurvival Bcl-xl actually triggered a complete megakaryocyte apoptosis and fail to produce platelets. On the contrary, platelet production was restored by deletion of proapoptotic Bcl-2 associated X protein and Bcl-2 homologous antagonist/killer, which also protected megakaryocytes from chemical-induced apoptosis and increased the platelet viability. Taken together, these data might suggest the need for controlled apoptosis.

Traditionally, 2 apoptotic pathways have been described: the cell-surface death receptor–dependent extrinsic pathway and the mitochondria-dependent intrinsic pathway. A way to induce a tightly controlled and restrained apoptosis process could also be via endoplasmic reticulum (ER) stress. ER stress is an easy and rapid way to maintain a control on the apoptosis level induced during the whole process. ER is the principal location for synthesis and folding of secreted, membrane-associated or organelle-targeted proteins. To assist in this folding process, lumen of ER constitutes a unique cellular compartment that encloses chaperones and several cofactors, such as ATP and Ca²⁺, as well as an optimal oxidizing environment. Any perturbation in the ER homeostasis can cause accumulation of misfolded proteins in its lumen promoting the ER stress.

In response, ER stress triggers an evolutionary conserved cellular response named unfolded protein response (UPR) intended to re-establish ER homeostasis and functions. However, prolonged UPR results in apoptosis. There are 3 described apoptotic signaling pathways triggered by ER stress that lead to the activation of caspase-3: the C/EBP homologous protein/GADD153 pathway, the cJun NH2-terminal kinase pathway, and the caspase-12 activation pathway.

Briefly, in unstressed conditions, chaperones such as glucose-regulated proteins 78 (GRP-78) bind and inhibit the effectors involved in UPR. Under stress conditions, GRP-78 recruitment away from those proteins results in both autophosphorylation and activation of protein kinase–like ER kinase and inositol-requiring transmembrane kinase/endonuclease 1 and activation...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATF-6</td>
<td>activating transcription factor 6</td>
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<tr>
<td>CTAP III</td>
<td>connective tissue-activating peptide III</td>
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<tr>
<td>elf2α</td>
<td>eukaryotic translation initiation factor 2α</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>GRP-78</td>
<td>glucose-regulated protein 78</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<td>SERCA3</td>
<td>sarco/endoplasmic reticulum Ca2+ ATPase type 3</td>
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<td>TPO</td>
<td>thrombopoietin</td>
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<td>Tun</td>
<td>tunicamycin</td>
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<td>UPR</td>
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of activating transcription factor 6 (ATF-6). Activation of these effectors induces (1) an overall decrease in mRNA translation, intended to reduce accumulation of unfolded proteins in the ER, and (2) transcriptional upregulation of genes involved in protein degradation and of those encoding for ER chaperones and folding enzymes which consequently increases the protein folding capacity of the ER.11 Inositol-requiring transmembrane kinase/endonuclease 1 is a type I transmembrane protein acting as an endoribonuclease and processing the X-box–binding protein-1 (Xbp1) mRNA. Spliced Xbp1 mRNA (Xbp1s) codes for Xbp1 translation factor, which upregulates the expression of chaperones such as GRP-78,12 whereas unspliced Xbp1 mRNA (Xbp1u) encodes a transcription factor that actively represses UPR target genes by binding and targeting Xbp1s for degradation.13 Therefore, the ratio of Xbp1s to Xbp1u is a useful measure of UPR induction. On ER stress, ATP-6 is cleaved in the Golgi apparatus, producing a transcriptionally active cytosolic fragment that goes to the nucleus to activate the transcription of UPR target genes, including Xbp1.14,15 The third ER stress member is protein kinase–like ER kinase, a type I transmembrane protein that activates the eukaryotic translation initiation factor 2α (elf2α) by serine phosphorylation,16 increasing translational initiation of the transcription factor ATF-4. ATF-4 regulates promoters of several genes including ER chaperones; genes involved in the ER stress–induced apoptosis pathway and others participating in the control of cellular redox status.17,18 The balance between these regulations seems important and explains why the role of protein kinase–like ER kinase pathway remains unclear because it has been described to participate to either cell death or cell protection.19–22 Nonetheless, elf2α-P was first described to play an antia apoptotic function and to enhance cell survival against ER stress–induced apoptosis.23

In the present study, we investigated the possibility that ER stress is induced during thrombopoiesis in relationship with the apoptotic-like phase of thrombopoiesis using different maturation models (megakaryoblastic cell line: MEG-01 and hematopoietic stem cells: CD34+)24,25 and immature pathological platelets.3 Our data suggest that induction of ER stress involved upstream of the apoptotic phase of megakaryocyte differentiation.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

ER Stress Induction During Maturation of Megakaryocytic Cell Lines

Megakaryoblastic cell-line maturation was induced using phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C,25 or thrombopoietin (TPO).26 Samples taken between day 1 and 3 were analyzed for ER stress and maturation markers at protein and mRNA levels. Calreticulin is an ER chaperon protein whose regulation has been described to be sensitive to some signal downstream of ATF-6. However, its increase is not observed in all cell types studied and was always 2 to 4 time less than those of GRP-78. Here, we used calreticulin as protein control as previously.3 Because no modification of its expression was observed during megakaryocyte maturation, although ER stress was induced, we assumed that in these sets of experiments no change will happen. As expected, the increase in Rap1b compared with the level of α-actin was similar (Figure 1 in the online-only Data Supplement) to those measured in comparison with calreticulin (Figure 2B). Levels of integrin GPIIia, a receptor for fibrinogen, and Rap1b protein, an ubiquitous Ras-related GTPase, which increased with megakaryocytic maturation, were used as maturation markers.27–29 Increased GRP-78 expression, elf2α phosphorylation, and Xbp1 mRNA processing were used as ER stress markers. PMA treatment resulted in an increase in GPIIia in DAMI, CHRF 288-11 and in MEG-01 cell lines (Figures 1A and 1B and 2A and 2B), and increased Rap1b expression was observed in MEG-01 cells confirming their maturation.24,25 PMA also induced ER stress as indicated by increase in (1) GRP-78 expression after either 1 day (CHRF 288-11 and MEG-01) or 2 days (DAMI) of treatment (Figures 1A and 2A and 2A), (2) phospho-elf2α in MEG-01 cells (Figure 2A and 2B), and (3) Xbp1 mRNA processing (Xbp1s) in MEG-01 cells (Figure 2C). Similarly, TPO treatment for 2 days of DAMI cells resulted in mild maturation (increased GPIIia expression), associated with a higher GRP-78 expression (Figure 1C). Of interest, in PMA-treated MEG-01 cells, most of the ER stress–induced Xbp1s was observed after 2 days of treatment, whereas no significant variation was observed after 1 or 3 days of treatment (Figure 2C). Taken together, these results sustained that ER stress is induced during megakaryocytic maturation.

Effect of Tunicamycin-Induced ER Stress Response on DAMI and MEG-01 Cell Maturation

To determine whether ER stress induction was involved in megakaryocytic maturation, we analyzed the effect of direct induction of ER stress in DAMI and MEG-01 cells. Calcium homeostasis is strictly maintained in the ER. Any change in this homeostasis results in ER stress.30–32 Usually, thapsigargin, an inhibitor of intracellular calcium pumps, is frequently used to induce ER stress. In this work, we have not used thapsigargin because we believed these calcium pumps and calcium homeostasis could be associated with the maturation process. Therefore, cells were treated for 24 hours
with 1 or 4 µg/mL of tunicamycin (Tun), a N-glycosylation inhibitor that is also commonly used to induce ER stress, and expression of both ER stress and maturation markers was analyzed at protein and RNA levels. Similar results were observed for both DAMI and MEG-01 cells (Figure 3). As expected, Tun treatment increased the expression of ER stress markers, that is, GRP-78 (Figure 3A) and Xbp1s (Figure 3B and 3C). ER stress induction was followed by an upregulation of expression in GPIIIa and Rap1b proteins (Figure 3A). The same treatment also increased the amount of connective tissue-activating peptide III (CTAP III) messengers that code for chemokine receptor that is induced during maturation in PMA-treated DAMI and CHRF 288-11 cells. CTAP III was increased from 100% in untreated cells to 163±53% and 180±68% for DAMI and 142±25% and 190±25% for MEG-01, in the presence of Tun 1 and 4 µg/mL, respectively (Figure 3B and 3C).

Effect of Sarco/Endoplasmic Reticulum Ca2+ ATPase Type 3b Overexpression–Induced ER Stress in MEG-01 Cell Maturation

As we previously observed that increase in sarco/endoplasmic reticulum Ca2+ ATPase type 3 (SERCA3) expression in human embryonic kidney-293 cells resulted in some level of ER stress,60 we genetically modified MEG-01 cells to express SERCA3 and to slightly enhance their level of ER stress. First, different SERCA3 isoforms were nucleofected in MEG-01 cells; SERCA3a did not produce GRP-78 expression, both SERCA3b and SERCA3f overexpression in MEG-01 cells led to ER stress, as illustrated by the higher level of GRP-78 expression measured 24 hours after nucleofection.
To further ascertain the relationship between SERCA3 expression, ER stress, and maturation, a single site mutation was conducted to delete the autophosphorylation site (Asp 351) of SERCA3b that is necessary for Ca\textsuperscript{2+} transport (Figure IIC in the online-only Data Supplement). The resulting SERCA3b\textsubscript{D351A} isoform was expressed in MEG-01 cells. Transfected wild-type SERCA3b and SERCA3b\textsubscript{D351A} MEG-01 cells were selected and tested for ER Ca\textsuperscript{2+} storage (Figure 4A and 4B). Wild-type SERCA3b overexpression resulted in a higher Ca\textsuperscript{2+} release in response to thapsigargin and ionomycin, compared with both controls and SERCA3b\textsubscript{D351A} expressing cells, confirming its effect on the ER Ca\textsuperscript{2+} storage and the nonfunctionality of the mutated isoform. Then, these cells were analyzed for SERCA3b, GPIIIa, and GRP-78 expression, ER stress, and maturation, a single site mutation was conducted to delete the autophosphorylation site (Asp 351) of SERCA3b that is necessary for Ca\textsuperscript{2+} transport (Figure 4C and 4D); both GRP-78 and GPIIIa expressions were increased in MEG-01 cells expressing wild-type SERCA3b compared with control or with cells expressing SERCA3b\textsubscript{D351A}. This was also confirmed at mRNA level, where CTAP III expression was higher in these cells than in controls or in MEG-01 cells expressing SERCA3b\textsubscript{D351A} (Figure 4E and 4F).

**ER Stress–Induced Apoptosis Activation Is Involved in MEG-01 Cell Maturation**

Caspases-3 and -9 have been reported to play a functional role in platelet production because inhibitors of both caspases blocked the proplatelet formation.\textsuperscript{2} Caspase-9 is an initiator caspase involved in the initial steps of apoptosis. Procaspase-9 (47 kDa) is cleaved in active caspase-9 (37 kDa) that further processes other caspase members, including caspase-3.\textsuperscript{35} Using an antibody that recognizes both forms of caspase-9, we observed that 48 hours of PMA treatment of MEG-01 cells resulted in a significant increase of the active form and a decrease in the inactive procaspase (Figure 5A). Similarly, cleavage of poly(adenosine diphosphate-ribose) polymerase, a caspase-3 substrate,\textsuperscript{2} was found to be increased under PMA treatment (Figure 5B). These findings confirmed a functional activation of apoptotic events during megakaryocyte maturation.

To investigate whether the caspase activation is dependent on ER stress, we have examined the effect of salubrinal on the caspase activation in PMA-stimulated MEG-01 cells. Salubrinal, a cell permeant that selectively inhibits the PP1 complex, has been shown to prevent elf2\textalpha-P dephosphorylation, protecting the cells from ER stress–induced apoptosis.\textsuperscript{23} As expected, 20 \textmu mol/L of salubrinal decreased both caspase-9 and poly(adenosine diphosphate-ribose) polymerase cleavage by \textasciitilde60\% in PMA-stimulated MEG-01 cells (Figure 5A and 5B).

The effect of salubrinal was then analyzed on thrombopoiesis. Although salubrinal treatment of MEG-01 cells by itself did not modify the expression of Rap1b and GPIIIa and only resulted in a time-dependent increase in elf2\textalpha-P (Figure 5D), it significantly inhibited the PMA-induced expression of these maturation markers associated with a greater increase in elf2\textalpha-P (Figure 5C and 5E).

Similarly, MEG-01 and DAMI cells treated with PMA in absence or in presence of salubrinal 30 \textmu mol/L were assessed by microscopy or flow cytometry. Figure 5F displays microscopic image showing that only PMA alone increased size of MEG-01 and DAMI cells, respectively. This was also observed by flow cytometry (data not shown). We also observed that PMA treatment of MEG-01 and DAMI cells increased the expression of GPIIa and that cotreatment with salubrinal abolished this increase (Figure 5G and 5H). Treatment with salubrinal alone did not modify the expression of GPIIb (data not shown).

**ER Stress Induction in Pathological Immature Platelets**

To determine whether a similar mechanism might occur in physiological conditions, we analyzed the GRP-78 expression in platelets from 2 patients (P1 and P2) from the same family. These patients, characterized by a heterozygous R1308P substitution in the von Willebrand factor A1 domain, had a
history of severe thrombocytopenia, with circulating platelet agglutinates, consistent with an immature phenotype. These platelets were described as being produced from immature megakaryocyte, corresponding to the proplatelet formation phase. Platelet protein lysates from these patients were tested for GRP-78 expression and compared with normal samples (Figure 6A). Expression of GRP-78 was found to be higher in platelets from both patients than in controls, reaching 196±13% and 181±27% for P1 and P2, respectively (Figure 6B).

Figure 4. Effect of sarco/endoplasmic reticulum Ca²⁺ ATPase type 3b (SERCA3b)-induced endoplasmic reticulum (ER) stress on MEG-01 cell maturation. MEG-01 cells that were transfected with empty plasmid (0), wild-type (WT), or nonfunctional mutant (Mut) SERCA3b isoforms, were selected over a week, loaded with Oregon green 488 BAPTA1-AM, and treated in absence of calcium (EGTA, 100 µmol/L) with 1 µmol/L thapsigargin (Tg) and 50 nmol/L ionomycin (iono). Typical mean of fluorescent intensity corresponding to the Ca²⁺ mobilization from internal store normalized for each cell to the intensity measured before Tg+iono addition (A). Histograms showing the means±SEM (n=5) of the increase in [Ca²⁺]i in response to Tg+iono as the percent of integrated Ca²⁺ release (B). Protein lysates from the same cells were immunoblotted for the expression of GPIIb, SERCA3b, glucose-regulated protein 78 (GRP-78), calreticulin (CRT), and GPIIb and quantified (D). Reverse transcription polymerase chain reaction showing the expression of connective tissue-activating peptide III (CTAP III), SERCA3b, SERCA2b, and GAPDH using 15 ng of total RNA from 0, WT, or Mut and relative quantification of CATP III, respectively (E and F). Results are representative of 4 independent experiments.

Figure 5. DAMI and MEG-01 cells were treated or not with 10 nM PMA in presence or absence of 30 µM salubrinal (Sal) for 2 days. Thirty µg of protein lysates from MEG-01 cells were analyzed for the expression of markers of ER stress, apoptosis, and maturation. Salubrinal treatment decreases ER stress-induced apoptotic markers: caspase-9 activation (A) and PARP cleaving (B) and ER stress-induced maturation markers (C, D & E), *significance (P<0.5) differences compared to level of expression after 2 days of culture in presence of PMA. Size was observed by microscopy (F) Scale bar = 100µm (zoom window ×3.25) and GPIb expression were assessed by flow cytometry. The level of GPIb expression observed in untreated cell (white square) or treated with PMA (black square) or PMA + Sal (grey square) are displayed as mean of fluorescence intensities (MFI) compared to untreated cells, for DAMI and MEG-01 cells, respectively (G and H). The results are representative of 3 independent experiments. ELF2α indicates eukaryotic translation initiation factor 2α.

Figure 6. A. Effect of ER stress on DAMI and MEG-01 cell maturation. MEG-01 cells were transfected with empty plasmid (0), wild-type (WT), or nonfunctional mutant (Mut) SERCA3b isoforms, selected over a week, loaded with Oregon green 488 BAPTA1-AM, and treated in absence of calcium (EGTA, 100 µmol/L) with 1 µmol/L thapsigargin (Tg) and 50 nmol/L ionomycin (iono). Typical mean of fluorescent intensity corresponding to the Ca²⁺ mobilization from internal store normalized for each cell to the intensity measured before Tg+iono addition (A). Histograms showing the means±SEM (n=5) of the increase in [Ca²⁺]i in response to Tg+iono as the percent of integrated Ca²⁺ release (B).
Figure 6. Endoplasmic reticulum (ER) stress in immature pathological platelets. Platelet protein lysates isolated from 3 normal donors (C1–C3) and 2 patients (P1 and P2) were immunoblotted for glucose-regulated protein 78 (GRP-78) and calreticulin (CRT; A). For quantification purposes, patients gave blood samples at least 4 times along 2 years, and the mean value for normal platelets (Ctl) was arbitrarily taken as 1 for each experiment. Results have been corrected for CRT and are expressed as the mean±SEM (B). The results represent 4 independent experiments.

ER Stress Induction During CD34+ Maturation

Finally, induction of ER stress during megakaryocytic maturation was confirmed using hematopoietic stem cells (CD34+ cells isolated from cord blood or after leukapheresis) treated with 10 ng/mL of TPO. Samples taken between day 6 and 16 were analyzed for expression of maturation and ER stress markers at protein and mRNA levels. Typical Western blots demonstrating protein expression in megakaryocytes cultured for 8 to 14 days are shown in Figure 7A. The expression of GRP-78 was transiently upregulated in cultured human megakaryocytes, starting at 31±11 at day 8 of culture, reaching 108±15% at day 11 to drop to 67±17% at day 16, respectively (Figure 7B). The expression of GPIIIa was slightly delayed, starting at 17±3% at day 8, increasing (49±8% on day 11) to reach a plateau at day 14, and it was still measured at 94±9% at day 16. Similar results were obtained at mRNA level, and Figure 7C shows typical amplification for both maturation and ER stress markers (CTAP III and Xbp1, respectively) in megakaryocytes cultured for 6 to 11 days. GAPDH amplification was used to control RNA amounts. Here, we observed that CTAP III mRNA expression was also upregulated along the treatment, confirming the maturation of megakaryocytes. Xbp1 mRNA splicing analysis revealed that ER stress was also transiently induced, and most of Xbp1s was observed for 10 days of culture.

As described for MEG-01 cells, salubrinal addition to TPO-cultured megakaryocytes blocked expressions of both Rap1b and GPIIIa that were reduced to 32±5% and 43±9% of those observed in TPO-stimulated megakaryocyte, respectively (Figure 7D). Such treatment results after 10 days in a significant decrease of mature megakaryocytes, as it is accessed by flow cytometry for CD41+ and CD42+ (Figure 7E).

To obtain further insight into the role of ER stress on megakaryocyte maturation, we studied the physiological effect of salubrinal on the final stages of platelet formation. As presented in the Figure 7F, the cotreatment of TPO-stimulated megakaryocytes with 10 and 20 µM salubrinal decreased the percentage of proplatelet-bearing megakaryocytes from 15.2±3.4% in control conditions without salubrinal to 3.7±0.3% and 1.8±0.7%, respectively, although treatment with 30 µM salubrinal completely blocked the proplatelet formation. When CD34+ cells were cultured for 10 days in presence of TPO, addition of salubrinal in the last 48 hours, a 22.3% decrease in the number of CD41+CD42+ was observed and the percentage of annexine positive cells in this population was decreased by 50% (data not shown). All these findings taken together suggest that ER stress induction is involved in CD34+ maturation phase and in the latter proplatelet formation process.

Discussion

Apoptotic signaling is required for thrombopoiesis and has to be restrained in the same time. Because ER stress is one possible way to induce a controlled apoptosis, our aim was to determine whether ER stress is involved during thrombopoiesis and whether it is acting upstream of the apoptosis process. Presence of ER stress was observed during in vitro maturation of both megakaryocytic cell lines and megakaryocytes from hematopoietic stem cells. Interestingly, ER stress induction seemed to be carefully regulated because it was transient. This was particularly clear for the splicing of Xbp1, which tended to occur at day 2 and 10 in PMA-treated MEG-01 cells and in CD34+ cultured in the presence of TPO, respectively. At protein level, the increase in expression of GRP-78 was also transient in cultured megakaryocytes. Its level reached a plateau after 11 days and then slightly decreased. It is described that thrombopoiesis in this system is partially synchronous; immature megakaryocytes were observed before day 7, whereas platelet shedding and caspase activation begins on day 10 and peaks at day 12. Therefore, it is possible that ER stress induction corresponds to the proplatelet formation stage. The observation of ER stress induction (similar to those observed in megakaryocytes cultured for 11 days) in immature platelets from von Willebrand disease patient further confirms that ER stress is transiently induced during thrombopoiesis and can remain high in immature platelets observed during pathological situations. It is however difficult to determine whether the presence of ER stress in these platelets signs only their immaturity or also an altered thrombopoiesis linked to the thrombocytopenia.

A more prolonged treatment of cell lines with PMA resulted in caspase-3 activation, but no further maturation was observed and cells were then oriented toward apoptosis. Actually, apoptosis illustrated by the cleavage of PMCA4b protein (data not shown) appeared after 2 days of treatment of CHRF 288-11 cells. It is possible that apoptosis is rapidly induced in these cells described to be more mature than DAMI or MEG-01.
cells, explaining the lower increase in expressions of GRP-78 and GPIIIa. Such results further confirm that a tidily regulated ER stress is necessary to guide the cell through differentiation rather than death. This paradigm is also supported by recent studies reporting that ER stress can, on one hand, trigger apoptosis in increasing number of pathological states, such as Wolcott-Rallison syndrome and associated diabetes mellitus. On the other hand, new functions of ER stress are now emerging. ER stress induction was described during the maturation of antibody-secreting β cells, but also during the differentiation of neuronal stem cells and primary keratinocyte cell lines.

However, maturation process is associated with the production of new proteins that will travel across the ER and may engender the need for a higher level of chaperones. To determine whether ER stress was part of the maturation process or only a collateral effect, we have tested the effect of direct ER stress induction using Tun in MEG-01 and DAMI cells or SERCA3b expression in MEG-01 cells suggests that it plays a direct role during this process. Of course, Tun treatment did not lead to a full maturation process because many proteins need glycosylation to be functional, in particular platelet integrins such as GPIIIb-IIIa. In our case, a prolonged Tun treatment did not produce a higher level of expression of maturation markers but activated the apoptosis program (data not shown), similar to what is observed for a prolonged PMA treatment. Apoptosis was also rapidly observed when Tun were added to CD34+ cells (data not shown). The expression of SERCA3b did not produce further maturation in MEG-01, and cells could be cultured for several weeks. These data suggest that modification of the ER calcium homeostasis is not sufficient to completely orientate the cell phenotype.

To further establish that ER stress–induced apoptosis is required in the maturation process, cultured MEG-01 cells and megakaryocytes were cotreated with salubrinal that protects cells from ER stress–induced apoptosis. The fact that salubrinal treatment inhibited caspase activation clearly indicated that ER stress activation occurs upstream to the apoptotic-like phase. Moreover, the salubrinal treatment completely abolished the expression of maturation markers in both cultured MEG-01 cells and megakaryocytes, further enlightening that ER stress–induced apoptosis is necessary. This was also confirmed by the fact that salubrinal inhibited in a dose-dependent manner the production of proplatelets from megakaryocytes.

Figure 7. Endoplasmic reticulum (ER) stress induction in thrombopoietin (TPO)-induced cultured human megakaryocytes. CD34+ cells were cultured in the presence of TPO for up to 16 days, and protein lysate and total RNA were obtained using Trizol reagent. Thirty micrograms of protein lysates were analyzed by Western blots for the presence of GPIIIa, glucose-regulated protein 78 (GRP-78), and calreticulin (CRT; A). Densitometric measurement of the relative expression of GPIIIa and GRP-78 proteins during CD34+ cell maturation. The values are expressed as means±SEM (n=3; B). Representative reverse transcription polymerase chain reaction (PCR; n=3) showing the amplifications of GAPDH, as internal control, connective tissue-activating peptide III (CTAP III), and X-box–binding protein-1 (Xbp1). Spliced Xbp1 (Xbp1s) and unspliced Xbp1 (Xbp1u) PCR products were discriminated after Pst-1 restriction (C). After 9 days of culture, salubrinal (30 μmol/L) was added or not to the medium for 48 hours. Protein lysates were analyzed by Western blots for the expression of GPIIIa, Rap1b, eukaryotic translation-initiation factor 2α-eIf2α-P, and calreticulin (CRT; D). CD34+ cells were cultured up to 10 days with TPO in presence or in absence of salubrinal. Cells were harvested after 6 or 10 days, and number of matured megakaryocytes were assessed by flow cytometry through expression of CD41 and CD42 (E). Similarly, after 9 days of culture, CD41+CD42+ megakaryocytes (MKs) were sorted and seeded at 2000 cells per well in 96-well plate in serum-free medium containing TPO and the indicated concentration of Salubrinal for 72 hours. Quantification of proplatelet MK was performed as previously described. Images were obtained using a Zeiss inverted microscope at a magnification of ×40 (F). The results represent 3 independent experiments performed in triplicate wells.
Taken together, these data indicate not only that ER stress occurred during thrombopoiesis but that it also plays a crucial role in it, activating the apoptotic-like phase. It seems that a tightly controlled ER stress is necessary to regulate the apoptosis signaling pathway that orients the fate of cells to either maturation or death.

Acknowledgments

J. Enouf and R. Bobe designed the research. J.J. Lopez and R. Bobe analyzed data and wrote the manuscript. J.J. Lopez, C. Chaabane, A. Polidano, and S. Dally performed experiments. P. Nurden collected patient’s platelet and helped with manuscript correction. A. Palazzo, K. Lebozec, and N. Debili performed CD34+ experiments. L. Albaran helped with MEG-01 cell experiments.

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Disclosures

None.

References


Significance

Thrombopoiesis is a complex mechanism that is still poorly understood. Recently, a role for apoptosis signaling pathways was controversial because some publications showed induction of proapoptotic proteins, whereas others established the need to restrain apoptosis pathways. Here, we showed that endoplasmic reticulum stress–induced apoptosis signaling is involved and required in the maturation process and might be the missing link that controlled both induction and reduction of apoptosis signal.
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Supplemental Figure I- Quantification of Rap1b compared to α-actin.
MEG-01 cells were cultured in the presence of 10 nM PMA for up to 3 days. 30 µg of protein lysates were immunoblotted to detect the presence of Rap1b and α-actin. The mean value of cells treated for 2 days was arbitrarily taken as 100%. The results are representative of 3 independent experiments.
Supplemental Figure II- Effect of SERCA3 on ER stress induction in MEG-01 cells.
A. MEG-01 cells were transfected or not with 10 µg pcDNA3.1 plasmids empty (Ctl), coding for GFP (GFP) or different isoforms of SERCA3 (SERCA3a, SERCA3b and SERCA3f), and harvested after 24h. Protein lysates were used for western blottings for SERCA3, GRP-78 and CRT expression. B. Mean values of GRP-78 relative expression quantifications. GRP-78 relative expression in Ctl was arbitrarily taken as 1. Results are representative of 5 independent experiments. C. cDNA sequences showing wild type SERCA3b and mutated SERCA3b wherein the Aspartic acid 351 (GAC) is changed into an Alanine (GCC).
Materials and Methods

Materials

Thrombopoietin (TPO), phorbol 12-myristate 13-acetate (PMA), tunicamycin (Tun), apyrase Grade VII, prostaglandin E1 (PGE-1), and bovine serum albumin (BSA) were from Sigma-Aldrich Chimie (Lyon, France). Salubrinal was from Tocris (Bristol, UK). Trizol™ solution was supplied by Invitrogen, Carlsbad, CA, USA. Anti-Caspase-9 antibody was from Merck Millipore (Billerica, MA, USA). Anti-GRP78 and FITC-conjugated anti-CD42b antibodies were from Becton Dickinson Biosciences (San Jose, CA, USA). Anti-GPIIIa antibody was a gift from Dr. D. Pidard (Paris, France). Anti-Rap1 and anti-eIF2α-P(Ser⁵¹) antibodies were from Cell Signalling Technology (Danvers, MA, USA). Anti-calreticulin (CRT) and antipoly (ADP-ribose) polymerase (PARP) antibodies were from Novus Biological (Littleton, CO, USA). Anti-mouse and anti-rabbit peroxidase-conjugated antibodies were supplied by Jackson Immunoresearch (West Grove, PA, USA). Enhanced chemiluminescence detection system (ECL) was from Pierce (Cheshire, UK). Restriction enzyme Pst1 and GoTaq® Flexi DNA Polymerase were from Promega (Promega France, Charbonnieres, France). All other reagents were of analytical grade.

Patients and platelet samples. Platelets were obtained from two patients with VWD type 2B associated thrombocytopenia previously characterized¹. Patients gave blood sample at least 4 times along 2 years. The control samples came from hospital staff. Blood was collected on 3.8% sodium citrate (9:1 v/v). Platelet-rich plasma was prepared by centrifugation at 120 g for 10 min in the presence of apyrase (100 mU/mL) and PGE-1 (1μM) to minimize platelet activation. All donors gave informed and written consent according to the Declaration of Helsinki. These studies have been approved by our ethics committee (PROMOTION INSERM : 2006). Number of approval : RBM 01-14. Name of the study : Réseaux sur les maladies héditaires de la production et des fonctions plaquettaires.
**Megakaryoblastic cell culture** - Human megakaryoblastic cells (Dami, CHRF-288 11 and MEG 01) were grown as described in ², and cultured in serum-free medium containing 10 nM PMA, for up to 3 days of culture, in presence or absence of 30 µM Salubrinal, or 10 ng/mL TPO for 2 days, or 1 and 4 µg/mL Tun for 1 day.

CD34⁺ haematopoietic stem cells were obtained from the umbilical cord blood or from healthy donors undergoing leukapheresis, after informed consent in accordance with the Declaration of Helsinki. Precursor cells were separated over a ficoll-metrizoate gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway) to obtain an enriched fraction of mononuclear cells. CD34+ cells were purified using an immunomagnetic cell-sorting system according to the manufacturer’s protocol (AutoMacs; Miltenyi Biotec, Bergisch Gladbach, Germany) and cultivated in serum-free medium containing TPO (10 ng/mL) as previously reported ³. Samples were taken between days 6 and 17.

**Nucleofection of MEG-01 cells** - MEG-01 cell nucleofection was performed using 5µg of pmaxGFP™ and 5 to 10 µg of pcDNA3.1 plasmid encoding for human SERCA3a, SERCA3b or SERCA3f cDNA, as per the manufacturer’s instructions (Amaxa biosystems, Koln, Germany).

**Site-directed mutagenesis of SERCA3b** - SERCA3b autophosphorylation site Asp351 was mutated in an Ala using Stratagene’s Quikchange II XL site-directed mutagenesis kit and primers 5’-CCTCAGTCATCTGCTCCGCACGACCCGACGCTCACC-3’ and 5’-GGTGAGCGTGCCCCTGTGCTGCGAGCAGATGACTGAGG-3’, both containing a single mutation (in bold), as per the manufacturer’s instructions. The presence of the mutation and the integrity of the SERCA3b<sub>D351A</sub> cDNA were then confirmed by sequencing (Supplemental Figure 1C).
Total RNA and protein isolation. Total RNA and protein lysate were simultaneously prepared using Trizol™ solution, as recommended by the manufacturer, and aliquots were stored at -20°C until use.

RT-PCR. Total RNA (2.5 µg) was reverse transcribed using Ready-to-go™ kit (GE Healthcare, Aulnay sous Bois, France). Gene specific primers were used to amplify mRNA by PCR on a Mastercycler EPGradient S (Eppendorf, Hamburg, Germany) using the following conditions: Touch Down-PCR (TD-PCR) was performed for 10 cycles with annealing temperature decrement from 65 to 55°C. PCR was conducted for 20 cycles, each consisting of successive periods of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. GAPDH amplifications were used as internal RNA controls. The primers and procedures used to amplify mRNA for GAPDH and Xbp1 have been detailed previously ⁴ and primers used for CTAP III (5’-AAACCATGGTTAACCTGGCGAAAGGC-3’ (5’ sens) and 5’-AAGGATCCTAATCAGCAGATTTCATC-3’ (3’ antisens) were similar to those used in ⁵.

Analysis of Xbp1 mRNA cleavage - RT-PCR were conducted as in ⁴. Briefly, the 600 base pair amplified DNA products, encompassing the IRE1 cleavage site, were further purified and digested with the restriction enzyme PstI prior been visualized on ethidium bromide stained 2% agarose gels using a Pulnix CCD camera and Infinity-capt software (Torey, Marne-la-Vallée, France) and quantified using Image J (National Institutes for Health, USA).

Immunoblotting. Protein lysate (30 µg) was loaded on minigels, separated by SDS-PAGE electrophoresis and transferred electrophoretically onto nitrocellulose membrane. Membranes were blocked using 5% (w/v) fat free milk or 10% (w/v) BSA in 20 mM Tris-HCl (pH 8.2) containing 0.05% (vol/vol) Tween 20 and incubated with the indicated antibodies. After washing, the membrane strips were then incubated with a 1:10 000 dilution of horseradish-peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG prior detection using an
enhanced chemiluminescence detection system (ECL). Luminescence was visualized and quantified using G-Box (syngene, UK) and Image J software.

**Ca\textsuperscript{2+} measurements** - MEG-01 cells (10\textsuperscript{6}/ml) were loaded with 10µM of Oregon Green 488 BAPTA1-AM (Molecular Probe, Invitrogen, France) at 37°C for 45 min. Fluorescence emission (523 nm) changes in Ca\textsuperscript{2+} were measured after 1 µM thapsigargin and 50 nM ionomycin (Sigma-Aldrich) were added to deplete stored Ca\textsuperscript{2+}. Changes in calcium concentration were analysed using video-microscopy (Nikon Eclipse TE2000-U inverted confocal microscope). MetaMorph software was used to control the system and perform image analysis. Images were acquired every 2 to 5 sec. Ca\textsuperscript{2+} mobilisation was estimated as the integral of the rise in [Ca\textsuperscript{2+}]\textsubscript{i} above basal level for 160 sec after store depletion in the absence of external Ca\textsuperscript{2+} (EGTA 100µM).

**Flow cytometric analysis** - Cells were harvested using Versene solution and then were either directly incubated with FITC conjugated anti-CD42b antibodies for 20 minutes at room temperature. The reaction was stopped by adding PBS and the samples were analyzed in an Epics XL (Beckman Coulter, Villepinte, France) or in an Accuri C6 (Becton Dickinson) flow cytometer.

**Quantification of proplatelet** - Cultured megakaryocytes derived from CD34\textsuperscript{+} cells cultured 9 days, as described above, were harvested and plated in 96-well plates at a concentration of 2000 cells/well in serum-free medium containing TPO for 4 days in presence of salubrinal at the indicated concentration. Megakaryocytes displaying proplatelets were then quantified by enumerating 500 cells per well using an inverted microscope at a magnification of x200.

**Statistics.** All data are expressed as means ± SEM. The statistical significance was determined by performing multiple comparisons among independent groups of data in which one-way ANOVA followed by a Tukey multiple comparison analysis indicated the presence
of significant differences. P<0.05 and P<0.01 were considered significant and indicated by
(*) and by (**) respectively.

References