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Pro-apoptotic signaling induced by photo-oxidative ER stress is amplified by Noxa, not Bim

Tom Verfaillie [a,1], Alexander van Vliet [a,1], Abhishek D. Garg [a], Michael Dewaele [a], Noemi Rubio [a], Sanjeev Gupta [c], Peter de Witte [b], Afshin Samali [c], Patrizia Agostinis [a,*]

[a] Laboratory of Cell Death Research and Therapy, Department of Cellular and Molecular Medicine, KU Leuven, Belgium
[b] Laboratory of Pharmacology, KU Leuven, Belgium
[c] Apoptosis Research Centre, NUI Galway, Ireland

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ABSTRACT

Pro-apoptotic signaling instigated by endoplasmic reticulum (ER) stress is tightly governed by the BH3-only proteins like Noxa and Bim, which help trigger apoptosis, in part by inactivating mitochondria protecting proteins like Mcl-1. Bim/Noxa-based pro-apoptotic signaling has been implicated for various ER stressors but not yet for those causing "ER-focused" production of severe oxidative stress. In the present study we found that photo-oxidative (phox)-ER stress induced by hypericin-based photodynamic therapy is associated with activation of PERK (an ER sessile, stress sensor), robust induction of CHOP (a pro-apoptotic transcription factor) and induction of Bim and Noxa (accompanied by an eventual drop in Mcl-1 levels). Interestingly Noxa, but not Bim, contributed toward phox-ER stress induced apoptosis, regulated by PERK in a CHOP-independent, temporally-defined manner. These observations shed further light on complex signaling pathways elicited byphox-ER stress and vouch for directing more investigation toward the role of PERK in cell death governance.

1. Introduction

Loss of endoplasmic reticulum (ER) homeostasis leads to ER stress [1] , which can be induced by various pathophysiological insults, including oxidative stress [2–4]. ER stress activates a conserved signaling pathway called the UPR (unfolded protein response) [1,3,4], which is relayed/co-ordinated by three ER-sessile stress sensors i.e., ATF6 (activating transcription factor 6), IRE1 (inositol requiring enzyme 1) and PERK (PKR-like ER kinase), each having its own "branch" of signaling cascades. Together, these sensors govern UPR, such that the switch between pro-survival and pro-death signaling is governed predominantly by the intensity of ER stress [1,3,4]. BCL-2 family members tightly regulate ER stress-mediated mitochondrial/intrinsic apoptosis [5]. Here, pro-death signaling is mainly mediated by various BH3 (BCL-2 homology 3)-only proteins (e.g., Noxa/Bim), which once activated cause loss of mitochondrial integrity by ablating mitochondria protecting proteins (e.g., Mcl-1) and triggering Bax/Bak activation-driven mitochondrial outer-membrane permeabilization culminating into mitochondrial apoptosis [5].

We recently characterized (to a certain degree) the "unique" nature of signaling pathways associated with "focused" oxidative-ER stress induced by hypericin-based photodynamic therapy (Hyp-PDT) [6,7]. Hyp-PDT is an established anticancer therapeutic modality which has been employed, previously, for clinical treatment of tumors [8] and was recently shown to induce immunogenic cancer cell death, *in vivo* [6,9,10]. Hyp-PDT employs a photosensitive drug called hypericin which predominantly associates with the ER membranes [6,9]. This ER-associated hypericin [11], when activated by light of a proper wavelength, evokes the production of reactive oxygen species (ROS) "focused" at the ER [12,13] (due to the short half-life and low diffusion range of ROS) [6,9,14]; thereby causing photo-oxidative (phox) stress-mediated disruption of ER-Ca^{2+} homeostasis [15] and induction of phox-ER stress [9,10]. Our analysis revealed that, phox-ER stress is characterized by up-regulation of various chaperones (e.g., GRP78/BiP) and the triggering of the two main UPR branches i.e., PERK-eIF2α-ATF4 branch (where, eIF2α: eukaryotic translation initiation factor 2α; ATF4: activating transcription factor 4) and the

Abbreviations: CHOP, C/EBP homologous protein; eIF2a, eukaryotic initiation factor-2a; ER, endoplasmic reticulum; GRP78, glucose regulated protein 78; Hyp-PDT, hypericin-based photodynamic therapy; ICD, immunogenic cell death; MEF, murine embryonic fibroblast; PERK, pancreatic ER kinase (PKR)-like ER kinase; Phox, photo-oxidative; ROS, reactive oxygen species; UPR, unfolded protein response.

* Corresponding author. Address: Cell Death Research and Therapy Laboratory, O&N I Herestraat 49, Box 802, 3000 Leuven, Belgium. Fax: +32 16 3 30735.
E-mail address: patricia.agostinis@med.kuleuven.be (P. Agostinis).

[1] These authors contributed equally to this work.

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IRE1α-XBP1 branch (where, XBP1: X-box binding protein 1) [7]. Interestingly we observed that the PERK branch, but not the IRE1α branch, mediated the pro-death signaling following phox-ER stress through activation of the pro-apoptotic transcription factor, C/EBP homologous protein (CHOP) [7]. All these events were observed to ultimately culminate into Bax/Bak-driven mitochondrial apoptosis [15].

Despite the recent insights gained into the phox-ER stress mediated pro-apoptotic signaling, there are still questions that remain unresolved. For instance, is there another PERK-mediated signaling pathway that contributes to pro-death signaling independent of CHOP following phox-ER stress? In a recent RNAi screen employed for deciphering the roles of BH3-only proteins during apoptosis, it was reported that ER stress-mediated apoptosis was associated mainly with Bim and Noxa-mediated signaling [16]. Here, Noxa has been reported to enable mitochondrial apoptosis by assisting in degradation of the pro-survival molecule, Mcl-1 [17]. Several kinds of ER stressors have been shown to induce Noxa-mediated pro-apoptotic signaling e.g., Bortezomib [18], Eeyarestatin I [18], lytic virus infection [19] and celastrol [20]. To this end we decided to investigate whether and how, following phox-ER stress, Bim or Noxa were regulating pro-death signaling.

In the present study we found that Hyp-PDT induces phox-ER stress associated with activation of PERK and CHOP and the BH3-only proteins Bim/Noxa. Moreover, Noxa, but not Bim, contributed toward phox-ER stress induced apoptosis, regulated by PERK in a, CHOP-independent manner.

2. Materials and methods

2.1. Reagents

Hypericin was synthesized from emodin anthraquinone according to Falk et al. [21], dissolved in DMSO and stored in dark conditions at −18 °C. Anti-β-Actin, total caspase-3, cleaved caspase 3, PARP, BiP, phospho-eIF2α, eIF2α, Bim, Puma, Chop, Mcl-1, anti-mouse HRP-linked (horse radish peroxidase) secondary and anti-rabbit HRP-linked secondary antibodies were obtained from Cell signaling technology (3 Trask Lane Danvers, MA 01923, USA), anti-Noxa antibody was obtained from Abcam for human-Noxa (330 Cambridge Science Park, Cambridge CB4 0FL, UK) or Sigma–Aldrich for mouse-Noxa (St. Louis, MO, USA). Sytox® green dead cell stain and was obtained from Invitrogen (5791 Van Allen Way Carlsbad, CA 92008, USA). Human PMAIP1 siRNA, Human BCL2L11 siRNA and Dharmafect 1 transfection reagent were obtained from Thermo scientific (Waltham, MA, USA).

2.2. Cell culture and treatments

Cell culture medium was prepared as follows: 500 ml DMEM (Dulbecco's modified eagle medium) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Gibco, Invitrogen, Carlsbad, CA, USA) and 10% (v/v) FBS (HyClone, ThermoFisher Scientific, Waltham, MA, USA). Cells were plated to achieve approximately 80% confluency on the day of treatment. Equal intracellular hypericin uptake in all cell lines used was analyzed by determining the red fluorescence (PE-channel, at 564–606 nm) on a FACScanto flow cytometer (BD bioscience, Erembodegem, Belgium).

2.3. Phox-ER stress induction by Hyp-PDT

MEFs were incubated with 200 nM hypericin for 2 h in serum-free DMEM in subdued light conditions (<1 μW/cm^2), followed by replacement of the medium with complete DMEM (with 10% FBS) after irradiation. T24 cells were incubated for 16 h with 150 nM hypericin in complete DMEM medium. Cell culture plates were placed on a plastic diffuser sheet approximately 10 cm above a set of seven L18W30 fluorescent lamps (Osram). The fluence rate of the lamps at the surface of the diffuser was 4.5 mW/cm^2 as measured with an IL 1400 radiometer (International Light, Newburyport, MA, USA). Unless differently specified, standard irradiation time was 6.5 min for both MEF and T24 cells, equivalent to a total light dose of 1.75 J/cm^2.

2.4. qRT-PCR

Total RNA was extracted using the PureLinkTM RNA Mini Kit (Life Technologies, Carlsbad, CA, USA). Total RNA (2 μg) was reverse-transcribed to cDNA using the Superscript III reverse transcription kit (Life Technologies). To determine the induction of UPR target genes, cDNA products were mixed with Brilliant III ultra-fast master mix (Agilent) and 20 × TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA/Integrated DNA Technologies, Coralville, Iowa, USA) and subjected to 40 cycles of PCR in a 7500 StepOnePlus instrument (Applied Biosystems). The relative quantity of each target transcript was normalized to GAPDH and relative expression was evaluated with $\Delta\Delta C_T$ method. Primer sets used were from Applied Biosystems and are summarized in Supplementary Table S1.

2.5. Supplementary materials and methods

For analysis concerning cell death, membrane depolarization, orthotopic AY-27 bladder tumor model, *in vivo* PDT treatment, transmission Electron Microscopy, siRNA transfection, western blotting and statistical analysis, please consult Supplementary Data.

3. Results

3.1. Phox-stress directed toward the ER activates robust signs of ER-stress and associated signaling

Phox-stress induced by *in vitro* Hyp-PDT treatment of cancer cells (T24 human bladder carcinoma) (Fig. 1A) and murine embryonic fibroblasts (MEFs) (Fig. S1A) elicited up-regulation of transcripts of several UPR-associated genes-a well-established marker of ER stress induction [7,22]. Analysis of the total RNA from Hyp-PDT treated cancer cells or MEFs followed by qRT-PCR revealed up-regulation of gene transcripts for ER chaperones like BiP/GRP78 and p58IPK, ERAD component like HERP and transcription factors like ATF4 and CHOP (Figs. 1A and S1A). The extent to which these transcripts were up-regulated depended on the cell type. However, among all these UPR-related transcripts the pro-apoptotic transcription factor CHOP was most robustly induced by Hyp-PDT induced phox-stress, irrespective of the cell type under consideration (Figs. 1A and S1A). We further confirmed the up-regulation of CHOP and GRP78 transcripts (two important ER stress effector molecules) on the protein level (Figs. 1B and S1A). After Hyp-PDT treatment a time-dependent accumulation of both CHOP and BiP was observed (Figs. 1B and S1A). In addition, Hyp-PDT induced a rapid phosphorylation of PERK's direct downstream target eIF2α (Fig. 1B). These observations clearly show that Hyp-PDT induces phox-ER stress *in vitro*.

Hyp-PDT has been used in preclinical and clinical set-up for tumor treatment previously [8,9], and its ER stress-inducing capabilities *in vitro* [6,9,10] were recently proposed to be behind its ability to induce antitumor immune responses *in vivo* [6]. This made us curious about whether Hyp-PDT also induced morphological

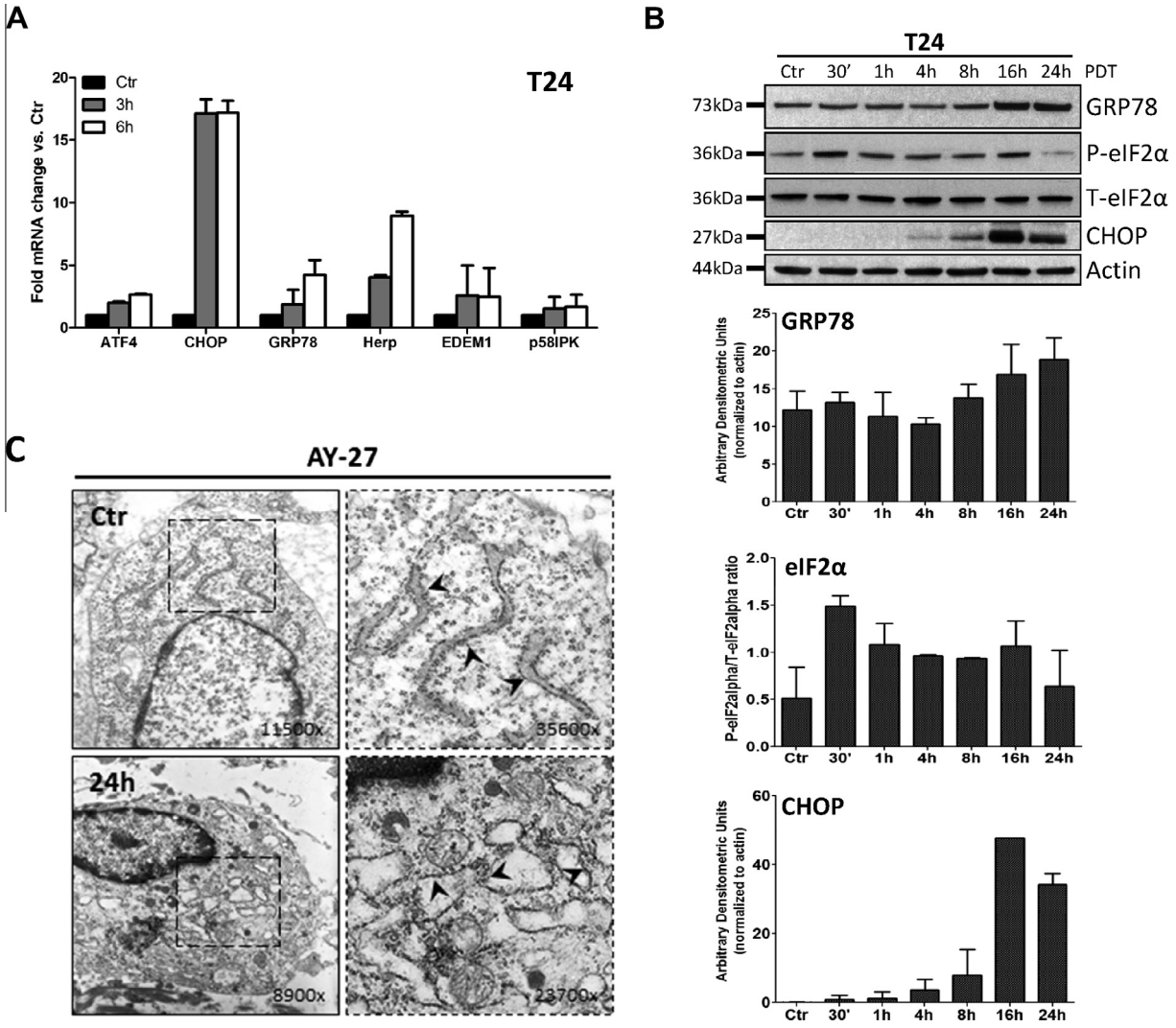

Fig. 1. Hyp-PDT induces ER stress *in vitro* as well as *in vivo*. (A) qRT-PCR analysis of total RNA isolated from Hyp-PDT treated T24 cancer cells for induction of the indicated UPR target genes. Graph represents the mean ± S.D. fold induction of two independent experiments performed in duplicate. (B) Top: western blot analysis of GRP78, phospho (P) and total (T) eIF2α and CHOP expression levels in T24 cells at the indicated times after Hyp-PDT treatment. Bottom: densitometric analysis of eIF2α phosphorylation and GRP78 and CHOP expression levels in Hyp-PDT treated T24 cells. Graph represents mean ± S.E.M. of three independent experiments. (**C**) Transmission electron microscopic images of sections from untreated (Ctr) and Hyp-PDT treated AY-27 bladder tumor tissue, 24 h after light irradiation of an orthotopic bladder cancer model in female fisher rats.

hallmarks of ER stress within a syngeneic, orthotopic transplantable tumor model *in vivo*. Hence, we analyzed sections of both untreated and Hyp-PDT treated rat bladder AY-27 tumors, established and treated as described previously [23], for morphological signs of ER stress *via* transmission electron microscopy. We found that, while untreated tumor tissue displayed a normal tubular ER network, tumor tissue treated with Hyp-PDT displayed massive ER expansion, a clear sign of ER perturbation (Fig. 1C).

Taken together these results demonstrate that Hyp-PDT induces phox-ER stress (*in vitro* and *in vivo*), associated with up-regulation of UPR-relevant molecules, *in vitro*.

3.2. Phox-ER stress up-regulates Bim and Noxa while eventually down-regulating Mcl-1 levels

Considering the recently discovered (tight) association between ER stress-induced apoptosis and up-regulation of the pro-apoptotic BH3-only proteins, Bim and Noxa [16,19,20], we decided to investigate the association of these molecules with phox-ER stress induced apoptosis. We first utilized qRT-PCR and analyzed the induction of Bim and Noxa in both cancer cells (Fig. 2A) and MEFs (Fig. S1B) after phox-ER stress. We observed an induction in Bim levels, varying depending on the cell type (Fig. 2A and S1B) but a consistent and cell type-independent induction of Noxa levels (Fig. 2A and S1B).

Moreover, these molecules were also induced at the protein level after phox-ER stress (Figs. 2B and S1B). Phox-ER stress induced a time-dependent up-regulation of the two isoforms of Bim i.e., Bim_{EL} and Bim_{L} [24] along with a robust up-regulation of Noxa (Figs. 2B and S1B). Interestingly, we observed that the protein levels of the anti-apoptotic protein, Mcl-1, showed an initial up-regulation (at 4–8 h post-treatment) followed by an eventual durable drop in levels later (at 16–24 h post-treatment) (Fig. 2B); concomitant with sustained up-regulation of Noxa protein levels (peaking at around 4–8 h post-treatment and nearly sustained until around 16 h) (Fig. 2B). Moreover, the increased accumulation of Bim isoforms as well as Noxa (Fig. 2B) and the decrease in Mcl-1 levels (Fig. 2B) coincided tightly with the processing/activation of

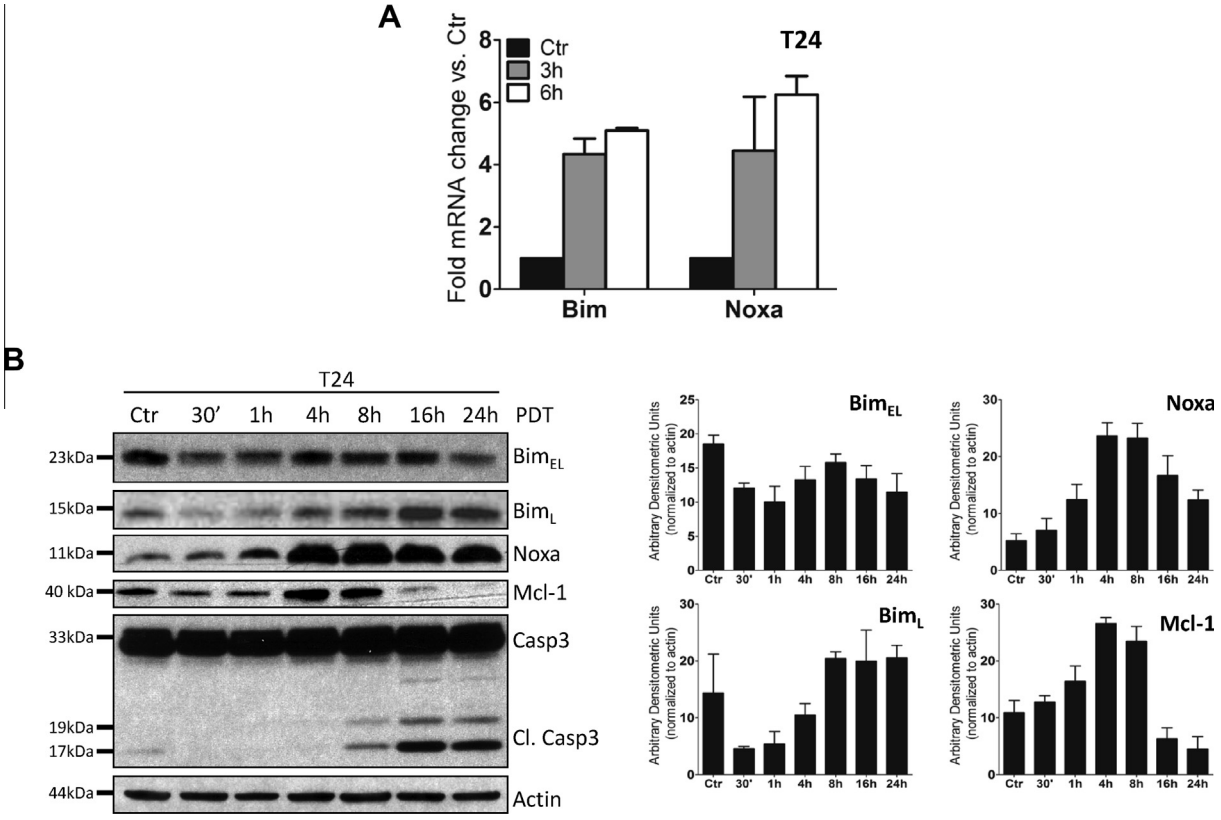

Fig. 2. Hyp-PDT induces the BH3-only proteins Bim and Noxa. (A) qRT-PCR analysis of total RNA isolated from Hyp-PDT treated T24 cancer cells for the BH3-only proteins Bim and Noxa. Graph represents the mean ± S.D. fold induction of two independent experiments performed in duplicate. (B) Left: western blot analysis of Bim (EL and L isoforms), Noxa, Mcl-1 and Caspase 3 (total and cleaved) expression levels in T24 cells after Hyp-PDT treatment. Right: densitometric analysis of Bim_{EL}, Bim_{L}, Noxa and Mcl-1 expression levels in T24 cells at the indicated times after Hyp-PDT treatment. Graph represents mean ± S.E.M. of three independent experiments.

pro-apoptotic caspase-3 (starting at 8 h and peaking at 16 h post-treatment) (Fig. 2B). This observation is in line with the reported predominant role of Noxa, in destabilizing Mcl-1 levels thereby inciting apoptosis [25].

Taken together these results reveal that phox-ER stress induced caspase-3 activation correlates strongly with the up-regulation of the pro-apoptotic proteins, Bim and Noxa, suggesting a role for these BH3-only proteins in the induction and amplification of apoptosis following phox-ER stress.

3.3. Noxa, but not Bim, contributes toward phox-ER stress induced apoptotic cell death

Considering that up-regulation of both Bim and Noxa correlated with phox-ER stress induced caspase-3 cleavage, it was imperative to understand, which of the two (or if both) are necessary for cell death in the current paradigm.

To this end, we silenced Noxa and Bim *via* siRNA in cancer cells, which resulted in a reduction of $\geqslant$50% in the levels of these proteins (Fig. 3A). Following treatment of these cells with phox-ER stress, we observed that reducing Noxa levels caused substantial hampering of caspase-3 processing (Fig. 3A), reduction of overall cell death (Fig. 3B) and concomitant resistance to loss of mitochondrial membrane potential (Fig. 3C). Interestingly, while Bim silencing affected caspase-3 processing to a small extent (Fig. 3A), yet it did not affect terminal apoptotic features like loss of mitochondrial membrane potential (Fig. 3C) or overall cell death (Fig. 3B).

In conclusion, Noxa, but not Bim, contributes toward phox-ER stress induced apoptosis.

3.4. ER stress sensor PERK, regulates Noxa levels in a temporally-defined fashion following phox-ER stress

PERK-induced signaling has been reported by us previously to be the dominant pro-apoptotic branch induced by phox-ER stress [26]. Since the transcription factor CHOP is a major pro-apoptotic target of the PERK pathway during the UPR [27], it was crucial to understand whether Noxa up-regulation was modulated downstream to CHOP induction in our ROS paradigm of ER stress.

Firstly, we evaluated the Noxa levels in $PERK^{+/+}$ and $PERK^{-/-}$ MEF cells after phox-ER stress. Interestingly, $PERK^{-/-}$ cells already displayed reduced basal levels of Noxa (more than 50% less) as compared to their WT counterparts (Fig. 4A) in untreated conditions. Moreover, following phox-ER stress while the $PERK^{+/+}$ MEFs clearly showed an increase in Noxa levels yet $PERK^{-/-}$ MEFs showed a temporally-defined (i.e., at 4 h post-treatment) inability to increase Noxa to the same overall high levels as could be reached by WT MEFs (Fig. 4A). Thus absence of PERK, compromised the ability of the cells to reach the full-extent of Noxa up-regulation following phox-ER stress.

Next, we analyzed the effect of phox-ER stress in $CHOP^{+/+}$ and $CHOP^{-/-}$ MEFs. Analysis of caspase-3 cleavage substantiated our previous results [7] about the pro-apoptotic role of CHOP following phox-ER stress such that as compared to their WT counterparts, the $CHOP^{-/-}$ MEFs showed markedly delayed activation of caspase-3 (Fig. 4B). However, under phox-ER stress there was no difference in overall levels of Noxa irrespective of CHOP's presence (Fig. 4B).

Taken together these results suggest that PERK might regulate phox-ER stress induced apoptosis *via* two independent pathways

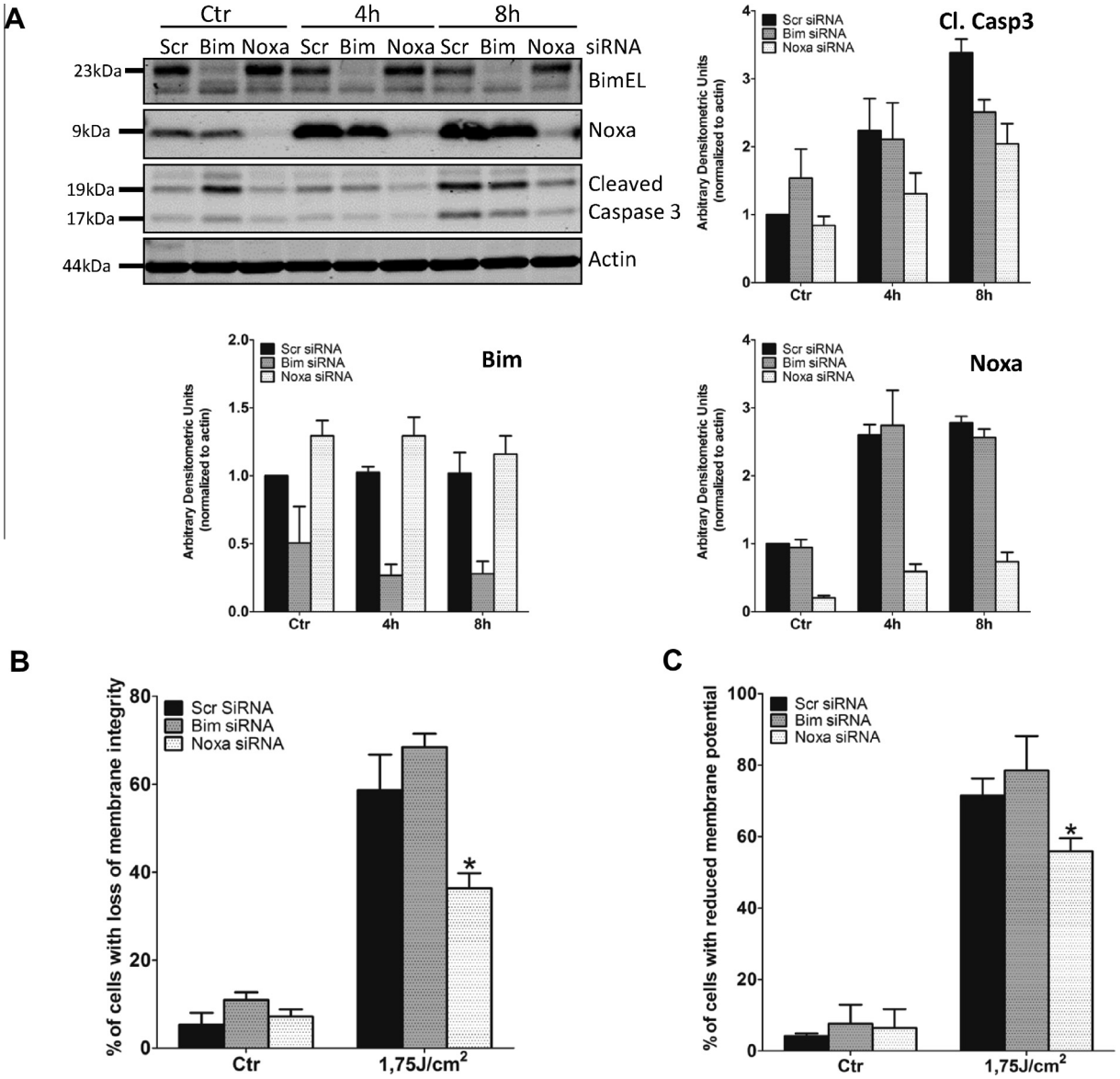

Fig. 3. Ablation of Noxa protects against Hyp-PDT induced apoptotic cell death. (A) Left: Western blot analysis of Bim, Noxa and cleaved Caspase 3 expression levels in T24 cells at the indicated times after Hyp-PDT treatment, 48 h after transfection with either Scr, Bim or Noxa siRNA. Right: densitometric analysis of cleaved Caspase 3 expression levels. Bottom: densitometric analysis of Bim and Noxa expression levels. Graphs represent mean ± S.E.M. of three independent experiments. (B) 48 h after transfection with scrambled (Scr), Bim or Noxa siRNA, T24 cells were Hyp-PDT treated and 16 h after irradiation, cell death was determined *via* LIVE/DEAD Near-IR Dead Cell Stain and subsequent flow cytometry analysis. Graph represents the mean ± S.D. of three independent experiments. * indicates $p < 0.05$ (C) 48 h after transfection with scrambled (Scr), Bim or Noxa siRNA, T24 cells were Hyp-PDT treated. 16 h after irradiation, loss of mitochondrial membrane potential was analyzed after incubation with 400 nM TMRM and the percentage of cells with reduced membrane potential were determined by flow cytometry. Graph represents the mean ± S.D. of three independent experiments. * indicates $p < 0.05$.

i.e., PERK-based CHOP induction and PERK-based up-regulation of Noxa.

4. Discussion

Various kinds of ER stressors were recently shown to utilize the BH3-only proteins like Bim and Noxa for pro-apoptotic signaling [16,19,20] but such information was not available for phox-ER stress. Results reported in the current study, taken together with our previous results [1,26] reveal a complex pro-apoptotic signaling cascade, with various amplification steps. Phox-ER stress induced by Hyp-PDT causes activation of the PERK branch which initially entails phosphorylation of PERK and subsequently phosphorylation of eIF2α, ultimately culminating into CHOP activation [6,7] (Fig. S2). Here, the PERK-based CHOP activation plays a predominant but not exclusive role in pro-apoptotic signaling [6,7] (Fig. S2). The results of this study show that PERK induces (in a CHOP-independent fashion) Noxa (paralleled by Mcl-1 down-regulation), which contributes to pro-apoptotic signaling possibly by facilitating Bax/Bak activation, an essential step for phox-ER stress induced mitochondrial apoptosis [17] (Fig. S2), as reported previously [1].

There are still several outstanding questions that need to be solved though. For instance, the mediator that links PERK and Noxa up-regulation under phox-ER stress is unknown (Fig. S2). A recent study suggested that this role can be played by PERK-based activation of ATF3/ATF4 under ER stress [18,28] – a point that needs urgent attention in near future. It is noteworthy that we have reported phox-ER stress to be associated with significant

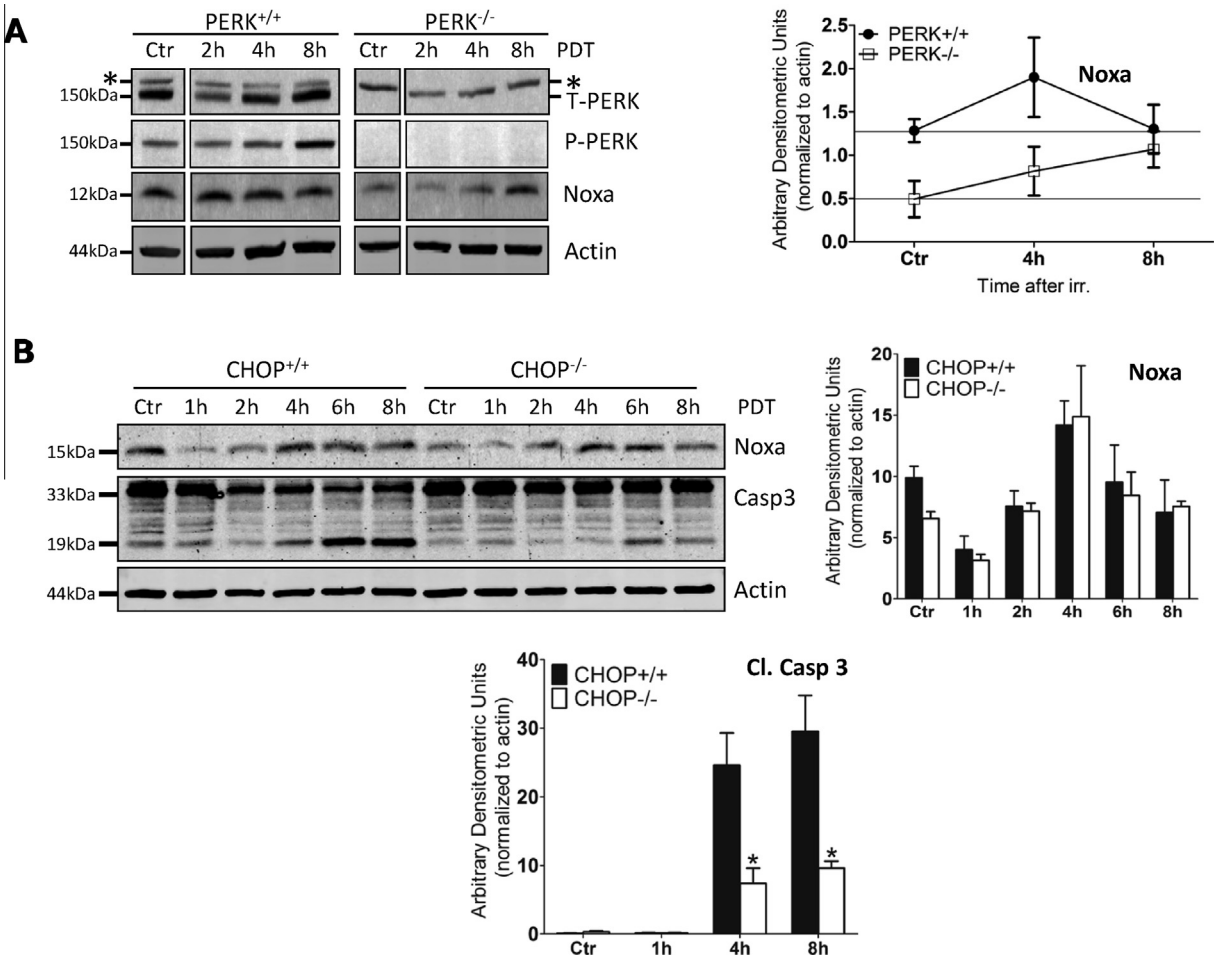

Fig. 4. Noxa expression levels are dependent on PERK. (A) Left: western blot analysis of PERK (phospho and total) and Noxa expression levels in PERK$^{+/+}$and PERK$^{-/-}$ MEFs, at the indicated times after Hyp-PDT treatment. * indicates a nonspecific band. Right: densitometric analysis of Noxa expression levels in PERK$^{+/+}$and PERK$^{-/-}$ MEFs at the indicated timepoints after Hyp-PDT treatment. Graph represents mean ± S.E.M. of three independent experiments. (B) Left: western blot analysis of Noxa and Caspase 3 (total and cleaved) expression levels in CHOP$^{+/+}$and CHOP$^{-/-}$ MEFs at the indicated timepoints after Hyp-PDT treatment. Right: densitometric analysis of Noxa expression levels in Hyp-PDT treated CHOP$^{+/+}$ and CHOP$^{-/-}$ MEFs. Bottom: densitometric analysis of cleaved caspase 3 expression levels in Hyp-PDT treated CHOP$^{+/+}$ and CHOP$^{-/-}$ MEFs. Graph represents mean ± S.E.M. of three independent experiments. * indicates $p < 0.05$.

up-regulation of ATF3 on the transcriptome-level [29]. Another open question concerns the lack of effect on phox-ER stress induced cell death observed upon the knockdown of Bim, a known target of CHOP [1,4]. Given that CHOP has a pro-apoptotic role in phox-ER stress and Bim up-regulation is blunted in CHOP$^{-/-}$ cells (data not shown), this observation is unexpected. However, it is interesting to note that heightened protein synthesis before restoration of proteostasis during ER stress, rather than up-regulation of pro-apoptotic BCL-2 family members, has been recently identified as a major pro-death mechanism mediated by CHOP and ATF4 [30].

Thus, while a better understanding of the complex signaling pathways activated following phox-ER stress, will require further analysis, the data presented in this and our previous work [7], strongly indicates that PERK is a master regulator of phox-ER stress and the associated signaling. The challenge in the future will be to integrate the emerging roles of PERK into a coherent but comprehensive picture.

Lastly, although the induction of robust ER stress and the associated UPR signaling in response to Hyp-PDT has been described extensively in our previous studies [6,7,9,10], here we report for the first time the ability of Hyp-PDT to induce morphologically recognizable signs of ER stress in a syngeneic, orthotopic bladder tumor model *in vivo*. This is especially important considering that Hyp-PDT induces "near-to-ideal" ER stress-mediated immunogenic cell death (ICD) in cancer cells [7,9,10,12,13,15]; and the above observation comforts the possibility of this ICD inducer causing phox-ER stress in tumor tissues, *in vivo*. Moreover, current results related to contribution of Noxa to pro-death signaling were similar to those obtained for Bortezomib [18], another renowned ICD inducer like Hyp-PDT [8,12,13]. Thus, in future it would be necessary to find whether Noxa up-regulation plays a crucial role in ICD regulation following Hyp-PDT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.107.

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