The PKCδ -Abl complex communicates ER stress to the mitochondria – an essential step in subsequent apoptosis

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Summary

Conditions that compromise protein folding in the endoplasmic reticulum trigger the unfolded protein response (UPR), which either restores proper protein folding or results in cellular demise through apoptosis. In this study, we found that, in response to ER stress in vivo and in vitro, PKCδ translocates to the ER where it binds to the tyrosine kinase Abl. Tyrosine phosphorylation and kinase activity of PKCδ are required for PKCδ binding to Abl in the ER. Moreover, we found that inhibition of PKCδ by the PKCδ-specific peptide inhibitor δV1-1 or by silencing of PKCδ reduces ER-stress-induced JNK activation and inhibits ER-stress-mediated apoptosis. Furthermore, the inhibitor of PKCδ kinase activity rottlerin blocks the translocation of the PKCδ-Abl complex from the ER to the mitochondria and confers protection against apoptosis. Thus, PKCδ communicates ER stress to the mitochondria by binding to ER-localized Abl. The PKCδ-Abl complex then translocates to the mitochondria, communicating ER stress to this organelle, thereby, triggering apoptosis.

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Key words: Protein kinase C, Apoptosis, Abl, Endoplasmic reticulum, Mitochondria

Introduction

Endoplasmic reticulum (ER) functions can be impaired by various intracellular and extracellular stimuli that lead to ER stress. Cells respond to ER stress by activating a signaling cascade termed the unfolded protein response (UPR), which results in the transcriptional upregulation of stress proteins and protein chaperones that enhance the protein folding capability of the ER (Boyce and Yuan, 2006; Ron and Walter, 2007; Xu et al., 2005). If these adaptive responses are insufficient to protect the cells from ER stress, UPR ultimately initiates apoptosis (Szegezdi et al., 2006b). Recently, it has become apparent that the ER plays a crucial role in mediating apoptosis. The ER serves as a site where apoptotic signals are generated through several pathways, including induction of the transcription factor CHOP (also known as DDIT3) (Oyadomari and Mori, 2004), proteolysis-induced activation of procaspase 12 (Nakagawa et al., 2000) and activation of the Jun N-terminal kinase (JNK) cascade through the ER membrane protein IRE1 (Urano et al., 2000). Importantly, ER-stress-induced apoptosis is associated with a variety of diseases, including neurodegenerative and myocardial diseases, stroke, and diabetes (Harding and Ron, 2002; Lindholm et al., 2006; Paschen and Doutheil, 1999; Szegezdi et al., 2006a). Moreover, pharmacological inhibition of ER stress confers protection from these injuries (Ozcan et al., 2006; Qi et al., 2004a; Qi et al., 2004b; Takano et al., 2007). Thus, ER stress can initiate cell death under pathological conditions. However, the mechanism responsible for ER-stress-induced cell death has not been completely elucidated.

Protein kinase C delta (PKCδ), a member of the PKC family, is involved in cell cycle regulation and apoptosis in a stimulus- and tissue-specific manner (Basu, 2003; Brodie and Blumberg, 2003). Inhibition of PKCδ using dominant-negative mutants of PKCδ, broad-spectrum inhibitors or a specific PKCδ antagonizing peptide can abrogate the apoptotic effects of a variety of stimuli (Murriel et al., 2004; Xia et al., 2007). The mechanisms by which PKCδ regulates cellular apoptosis has been studied in different systems, and may include proteolytic activation by caspase 3, tyrosine phosphorylation, association with specific apoptotic proteins and translocation of the activated PKCδ to the mitochondria (Basu, 2003; Brodie and Blumberg, 2003). However, whether PKCδ is involved in ER dysfunction is not known.

Previous studies demonstrated that PKCδ interacts with Abl, a non-receptor tyrosine kinase, under ionizing radiation and oxidative stress (Sun et al., 2000; Yuan et al., 1998). Moreover, Abl is found in the nucleus, the cytoplasm and the ER (Ito et al., 2001; Shaul, 2000), and in cells subjected to ER stress, Abl is targeted from the ER to the mitochondria, a step required for apoptosis (Ito et al., 2001). Thus, Abl seems to act as a communicator between the ER and mitochondria following ER stress.

Here, we investigated the possible involvement of PKCδ and Abl in the ER-stress-induced cell death pathway in cell culture and in an in vivo stroke model. We found that PKCδ interaction with Abl plays a crucial role in ER-stress-induced apoptosis by communicating ER stress to the mitochondria.

Results

PKCδ translocates to the ER following ER stress

One of the factors that contribute to the distinct roles of PKCδ is its translocation to diverse subcellular sites in response to various stimuli. For example, PKCδ translocates to mitochondria in response to UV radiation in human keratinocytes (Denning et al., 2002), TPA

in U937 cells (Majumder et al., 2000) and ischemia/reperfusion in hearts (Murriel et al., 2004). It also translocates to the nucleus in MCF-7 cells when exposed to ionizing radiation (Yuan et al., 1998) and in NG 1815 cells stimulated with dopamine (Gordon et al., 2001). Thus, we first determined whether PKCδ translocates to the ER under ER stress.

Neuro2a cells were treated with the ER stress inducer tunicamycin (Tm) that inhibits N-glycosylation. Western blot analysis revealed a significant increase in the level of PKCδ in ER-enriched fractions following 3 minutes of Tm treatment (a threefold increase, compared with control cells) (Fig. 1A). Since PKCδ has been reported to translocate to the mitocondria (Denning et al., 2002; Li et al., 1999; Majumder et al., 2000; Murriel et al., 2004), we also determined PKCδ levels in the mitochondrial fraction under ER stress conditions. The levels of PKCδ in mitochondrial fractions increased significantly only 3 hour after Tm treatment and remained high for at least 3 hours (Fig. 1A). The relative purity of the subcellular fractions was confirmed by immunoblotting with antibodies against the ER marker calnexin, the mitochondrial marker TOM20 and the cytosolic marke renolase (bottom panel in Fig. 1A). In addition, we confirmed that there was no change in total levels of PKCδ in control and Tm-treated cells (data not shown).

To further assess the subcellular distribution of PKCδ, confocal microscopy was performed to detect colocalization of PKCδ with the ER marker protein-disulfide isomerase (PDI) (Sitia and Molteni, 2004). Following 3 minutes of treatment with Tm or thapsigargin (Tg; an inhibitor of Ca-ATPase), colocalization of PKCδ (red) and PDI (green) increased (Fig. 1B, top panel; merged signals, yellow), as compared with control-treated cells. By contrast, there was only a minimal colocalization of PKCδ (red) and mitochondria marked with mitotracker (green) 3 minutes after Tm treatment; a greater localization of PKCδ towards the mitochondria was observed 3 hours after Tm treatment (Fig. 1B, bottom panel). These results

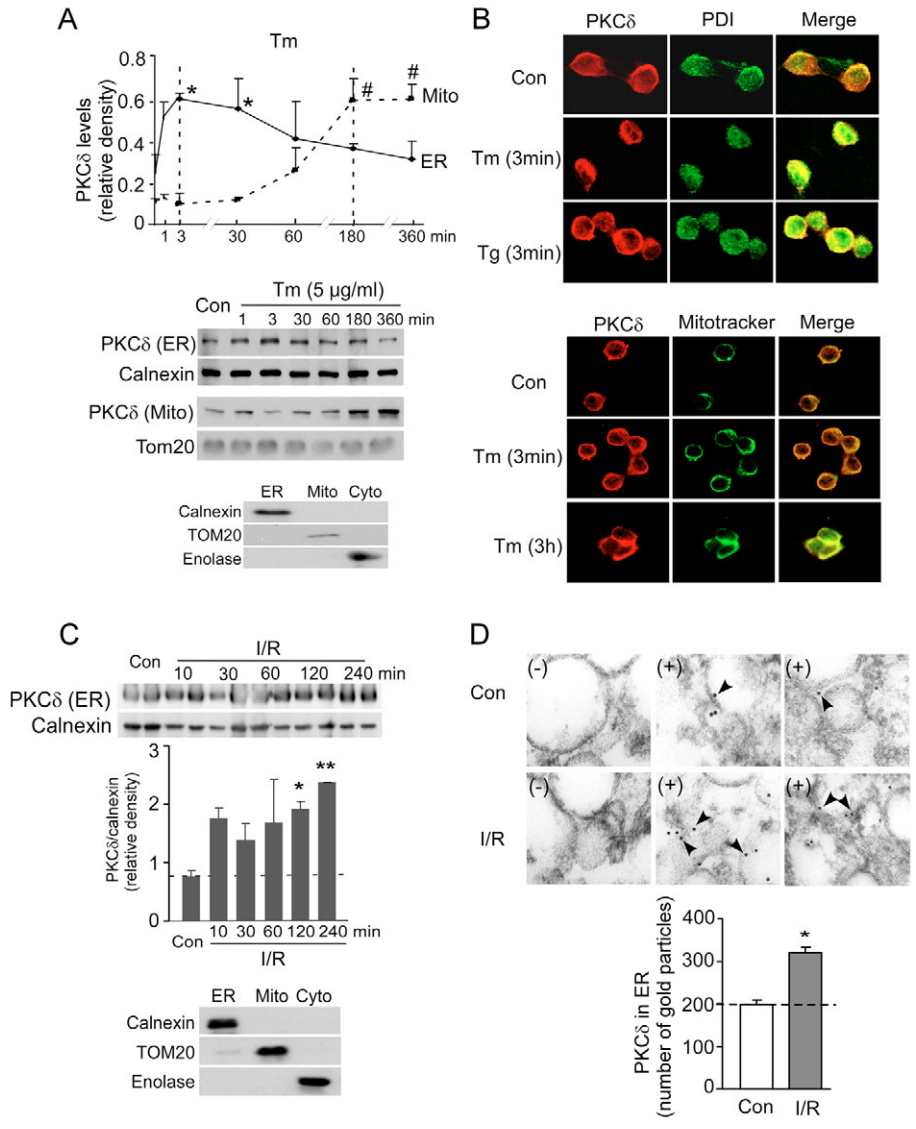

Fig. 1. PKCδ translocates to the ER following ER stress. (A) Neuro2a cells were treated with Tm (5 μg/ml). Cell lysates from the ER and mitochondria-enriched fractions were subjected to western blot analysis at the indicated times. (Top panel) Changes in the level of PKCδ in the ER and the mitochondrial fractions at the indicated time points. **P*<0.05 vs control in ER fractions, #*P*<0.05 vs control in mitochondrial fractions (*n*=3). (Centre panel) Representative western blot of PKCδ in the two fractions. Calnexin and TOM20 (markers of ER and mitochondria, respectively) were used as internal loading controls for quantification. (Bottom panel) The relative purity of the cellular fractionations was determined by the presence of calnexin, TOM20 and enolase (cytosolic marker). (B) Neuro2a cells were treated with Tm (5 μg/ml) or Tg (3 μM). (Top panel) Representative confocal microscopy image of PKCδ (red) and PDI (green), demonstrating increased colocalization (yellow) of PKCδ in the ER following 3 minutes of Tm or Tg treatment. Original magnification was ×60. The data are from three independent experiments. (Bottom panel) Representative confocal microscopy image of PKCδ (red) and mitotracker (green) after 3 minutes or 3 hours of Tm treatment. The data are from two independent experiments. (C) Rats were subjected to 2 hours MCAO followed by 10-240 minutes of reperfusion (I/R). Lysates from the penumbra area of the ipsilateral hemisphere of the rats brains were fractionated. (Top panel) ER fractions were subjected to western blotting using PKCδ antibody. Calnexin was used as an internal loading control for quantification. (Centre panel) Histogram depicting the amount of PKCδ associated with the ER in brain samples (PKCδ/calnexin). Data are expressed as the mean ± s.e. from results of four rats, **P*<0.05; ***P*<0.01, vs sham-operated rats. (Bottom panel) Confirmation of purity of fractionations by using the specific protein markers as described in Fig. 1A. (D) ER localization of PKCδ determined by immunoelectron microscopy. Representative electron microscopy image of PKCδ staining in the ER fractions from brains subjected to 2 hours MCAO followed by 4 hours of reperfusion (I/R). (magnification ×35,000). Samples were probed in the presence (+) or absence (–) of PKCδ antibody. Arrows indicate PKCδ-positive staining with gold particles. Quantification of gold particles associated with ER lumen are provided in the lower histogram. Five random fields of each section from three animals were counted. Data represent the mean ± s.e. from results of three animals per group, **P*<0.05 vs sham-operated rats.

suggest that an early response to ER stress induces PKCδ translocation to the ER followed by its translocation to the mitochondria.

Because ER stress occurs under a number of clinical conditions, we next determined whether PKCδ translocation to the ER could be observed in vivo. Stroke causes severe ER dysfunction and has been found to be an inducer of ER stress (Paschen, 2003; Paschen and Mengesdorf, 2005). Following a stroke model in rat, induced by a transient middle cerebral artery occlusion (MCAO), we observed that PKCδ translocated to the ER within 10 minutes of reperfusion, and that PKCδ remained in this fraction even after 4 hours of reperfusion (Fig. 1C). Subcellular fractionation was confirmed by probing with markers of each fraction (calnexin for ER-enriched fraction, TOM20 for mitochondria and enolase for cytosol) (Fig. 1C). To further confirm the translocation to the ER, we determined the presence of PKCδ by immunogold electron microscopy in brain samples collected following stroke. Immunogold labeling of PKCδ in ER-enriched fractions of rat brain following 2 hours ischemia followed by 4 hours of reperfusion increased by 40% as compared with the sham-operated group (Fig. 1D). These data demonstrate that stroke causes translocation of PKCδ to the ER.

ER stress induces apoptosis through a PKCδ-dependent mechanism

We have found previously that PKCδ translocation and subsequent function can be inhibited by δV1-1, a short peptide-translocation inhibitor (Chen et al., 2001; Murriel et al., 2004). Because we found that PKCδ plays a crucial role in the apoptotic process (Inagaki et al., 2003; Murriel et al., 2004), and severe or prolonged ER stress causes cell death mainly by apoptosis (Szegezdi et al., 2006b), we next investigated whether PKCδ is crucial for ER-stress-induced cell death. Activated JNK is a mediator of ER-stress-induced apoptosis (Ron and Walter, 2007), and ER stress is largely responsible for JNK activation (Urano et al., 2000). We therefore determined JNK activation and found a twofold increase in JNK activation in rat brains subjected to 2 hours of ischemia followed by 24 hours reperfusion and a sevenfold increase in cultured cells treated for 24 hours with Tm or 18 hours with Tg. Significantly, treatment with δV1-1, which inhibits translocation of PKCδ to the ER in vivo and in vitro (Fig. 2A), abolished the accumulation of activated (phosphorylated) JNK in both in vivo and in vitro (Fig. 2B,C). Moreover, following Tm and Tg treatment, δV1-1 inhibited phosphorylation of Jun (Fig. 2D), which executes apoptosis through the JNK cascade (Dunn et al., 2002). In the absence of ER stress, treatment with δV1-1 alone has no effect on JNK or Jun phosphorylation under both in vivo and in vitro conditions (data not shown). In addition, we determined the levels of two other ER-stress-related apoptotic molecules, CHOP and caspase-12. Stress-induced induction of CHOP and activation of caspase-12 were significantly inhibited ($P<0.05$) by δV1-1 treatment of rats subjected to 2 hours ischemia followed by 24 hours of reperfusion, and an inhibition by 20% was found in cultured Neuro2a cells (supplementary material, Fig. S1). Therefore, our results in vivo and in cultured cells strongly suggest that PKCδ participates in ER-stress-induced apoptotic signaling pathways mainly through the JNK pathway.

Since inhibition of PKCδ reduces ER stress and blocks ER-stress-induced apoptotic signals, we next examined the involvement of PKCδ in ER-stress-induced cell death. As shown in Fig. 2E, 30 hours after Tm or Tg treatments, Neuro2a cells showed significant TUNEL-positive staining, and inhibition of PKCδ by δV1-1 markedly decreased the number of TUNEL-positive cells. These data demonstrate that upon ER stress, Neuro2a cells undergo cell death, which is mediated by the activation of PKCδ.

PKCδ interacts with Abl in the ER following ER stress

We found that PKCδ activation and translocation to the ER takes place rapidly and transiently after induction of ER stress, and that the enzyme subsequently accumulates in the mitochondria. This indicates that, upon ER stress, translocation to the ER and not to the mitochondria is the first step in initiating the PKCδ-mediated apoptotic signaling cascade. We considered whether PKCδ interacts with specific proteins in the ER to enable the subsequent mitochondrial localization and apoptosis. Abl has been demonstrated to be one of the proteins that interact with PKCδ (Basu, 2003). Moreover, interaction of Abl with PKCδ seems to be crucial for apoptosis in response to oxidative stress and ionizing radiation (Sun et al., 2000; Yuan et al., 1998). Thus, we next determined whether Abl is essential for PKCδ function in the ER stress response. We first examined the level of Abl in the ER and in the mitochondrial fractions following Tm treatment. Consistent with a previous study (Ito et al., 2001), Tm treatment was associated with a decrease in Abl levels in the ER and an increase of this enzyme in the mitochondria (Fig. 3A), suggesting that ER stress causes Abl redistribution from the ER to the mitochondria. We also noticed that the time course of PKCδ translocation from the ER to the mitochondria (Fig. 1A) is similar to that of Abl (Fig. 3A). Thus, we determined whether translocation of PKCδ and Abl from the ER to the mitochondria is owing to their direct interaction. Analysis of anti-Abl immunoprecipitates by immunoblotting with anti-PKCδ antibody demonstrated a significant increase in the association of PKCδ with Abl in the ER fraction 30 minutes after Tm treatment (Fig. 3B upper panel). In reciprocal experiments, immunoblot analysis of anti-PKCδ immunoprecipitates using anti-Abl antibody confirmed the association of PKCδ with Abl (Fig. 3B, lower panel). Furthermore, the PKCδ-specific peptide inhibitor δV1-1 significantly inhibited the association of PKCδ with Abl in the ER fractions, compared with the TAT control treatment (Fig. 3B). The results suggest that ER stress induces the binding of PKCδ to Abl in the ER fraction. Under the same conditions, the mitochondrial fractions were also subjected to immunoprecipitations with anti-Abl and anti-PKCδ antibodies. Interestingly, a significant amount of the complex was found in mitochondria fractions after 3 hours, but not after 30 minutes following Tm treatment and, again, δV1-1 inhibited this interaction (Fig. 3B). These data suggest that under ER stress, PKCδ and Abl first interact in the ER and later translocate to the mitochondria, perhaps as a pre-formed complex.

We next determined whether PKCδ-Abl interaction occurs also in vivo in the rat MCAO stroke model. Consistent with the findings for cultured Neuro2a cells, 2 hours of ischemia followed by 24 hours reperfusion resulted in increased interaction of PKCδ and Abl in the brain penumbra area. Moreover, δV1-1 inhibited this association (Fig. 3C). These in vitro and in vivo data collectively support our hypothesis that stimuli associated with ER stress induce binding of PKCδ to Abl.

Interdependence of Abl and PKCδ in ER-stress response

To investigate the effect of Abl on PKCδ translocation to the ER, we reduced the cellular levels of Abl by transfecting cells with small interfering RNAs (siRNAs) targeting Abl (Fig. 4A). We found that translocation of PKCδ to the ER after a 3-minute treatment with

Tm was abolished when Abl levels were knocked down, compared with control-siRNA transfected cells (Fig. 4B, upper panel). By contrast, we did not find changes of PKCδ levels in the mitochondria at the corresponding time point (data not shown), but the levels were decreased after 3 hours of Tm treatment (Fig. 4B, middle panel). Moreover, confocal microscopy imaging analysis confirmed that there is little colocalization of PKCδ (red) and the ER marker PDI (green) following ER stress after Abl knockdown, compared with Tm-treated cells that had been transfected with control siRNA (Fig. 4C). These data strongly suggest that Abl is required for translocation of PKCδ to the ER.

We next determined whether PKCδ is required for Abl translocation to the mitochondria. Abl translocation to mitochondrial fractions occurred 3 hours after ER stress, and PKCδ knockdown completely abolished translocation of Abl to the mitochondria (Fig. 4D). These data indicate that PKCδ is required for the redistribution of Abl from the ER to the mitochondria following ER stress.

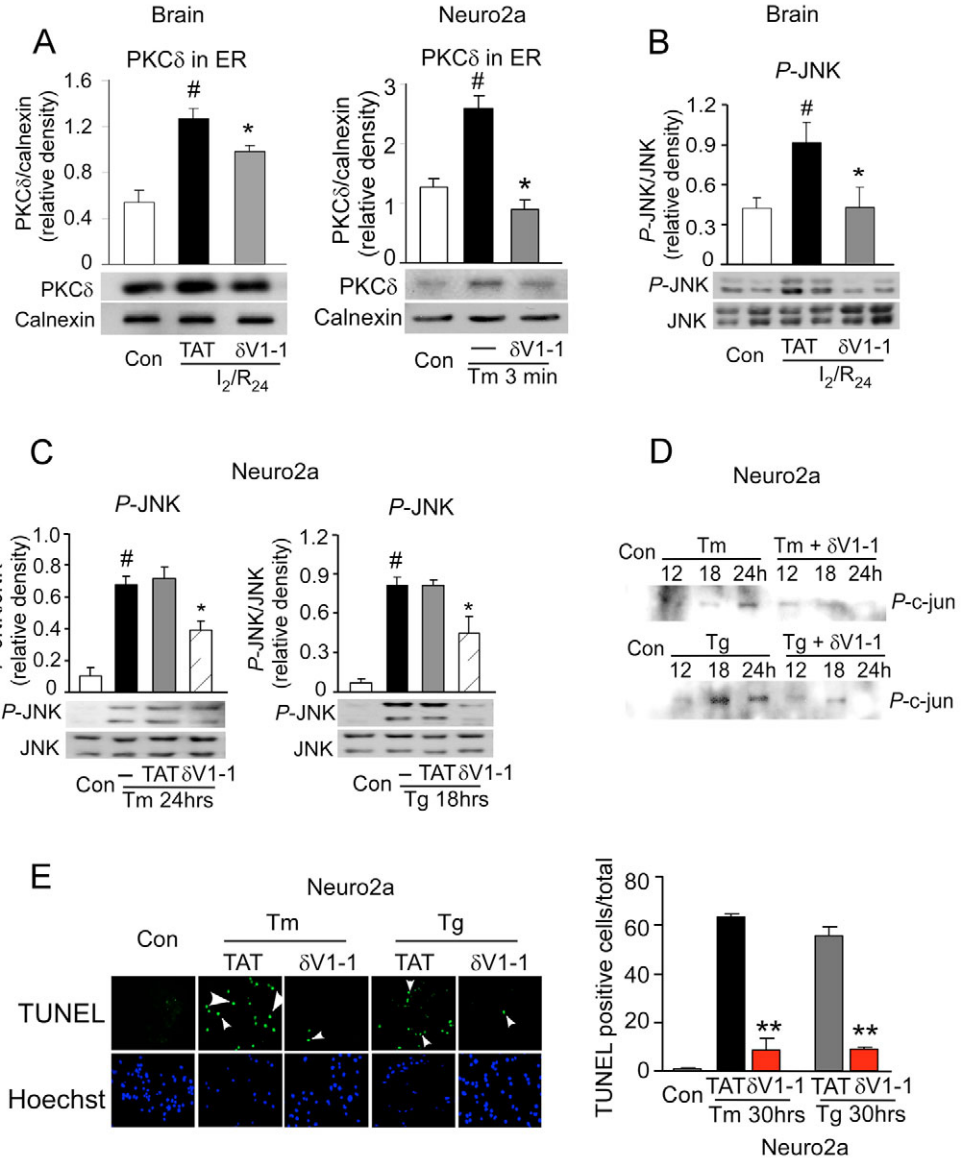

Fig. 2. δV1-1 inhibits ER-stress-mediated apoptosis. Rats were subjected to 2 hours MCAO followed by 24 hours of reperfusion (I2/R24). δV1-1 and TAT control (0.2 mg/kg) were injected intraperitoneally at the onset of reperfusion. Neuro2a cells were pre-treated with δV1-1 or TAT control (1 μM) for 15 minutes followed by Tm (5 μg/ml) or Tg (3 μM) treatment. (A) (Left panel) ER fractions were isolated from brain. (Right panel) After 3 minutes of Tm treatment, ER fractions were isolated from Neuro2a cells. The lysates were subjected to western blot analysis using anti-PKCδ antibody. Calnexin was used as internal loading control. (B) Induction of JNK phosphorylation was detected in total-cell lysates of rat brains. (C) Phosphorylation of JNK in total lysates of Neuro2a cells was detected by western blot after 24 hours of Tm (left) or 18 hours of Tg (right) treatment. (D) Phosphorylation of Jun, a downstream effector of JNK, was detected by western blotting at the indicated time points after Tm (upper) or Tg (lower) treatment. (E) TUNEL assay was carried out after 30 hours of Tm or Tg treatment. (Left panel) Representative data of three independent experiments. Original magnification was ×40. (Right panel) TUNEL-positive cells are expressed as a percentage of the number of total cells, as determined by staining with Hoechst dye. In the animal study, data are expressed as mean ± s.e. from results of six rats per group. $^{*}P<0.05$ vs TAT treatment; $^{\#}P<0.05$ vs sham-operated rats. In the cell culture study, the data are presented as mean ± s.e. of three independent experiments. $^{\#}P<0.05$ vs control, $^{*}P<0.05$ vs Tm treatment, $^{**}P<0.01$ vs TAT treatment.

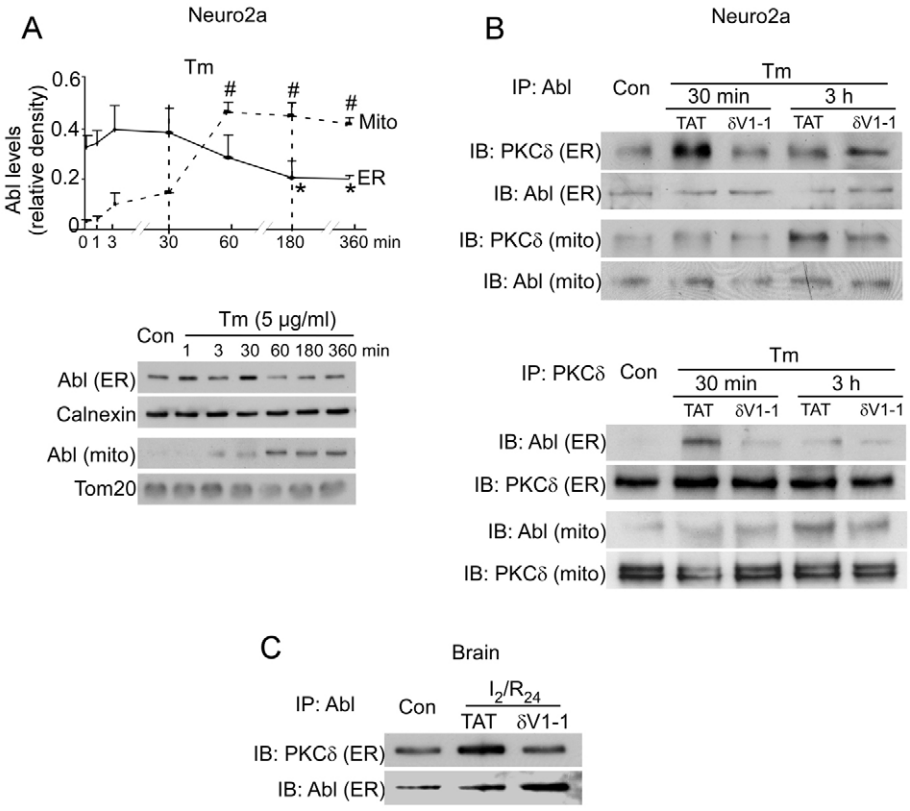

Fig. 3. ER stress induces the interaction of PKCδ and Abl. Neuro2a cells were pretreated with δV1-1 or TAT control (1 μM) for 15 minutes followed by Tm (5 μg/ml) treatment at the indicated time points. ER and mitochondrial (mito) fractions were then isolated. (A) The levels of Abl in the ER and mitochondria were detected by western blot. (Top) Graph depicting the levels of Abl in the ER and the mitochondrial fractions at the indicated time points. **P*<0.05 vs control in ER fractions; #*P*<0.05 vs control in mitochondria fractions. (Bottom) Representative western blot of staining for Abl in ER and mitochondrial fractions of two independent experiments. Calnexin (ER marker) and TOM20 (mitochondrial marker) were used as loading controls. (B) (Top) ER and mitochondrial fractions were subjected to immunoprecipitation (IP) with anti-Abl antibody and the immunoprecipitates were analyzed by immunoblotting (IB) with anti-PKCδ and anti-Abl antibodies at the indicated time points. Shown are representative data of four independent experiments. (Bottom) Immunoprecipitates obtained using anti-PKCδ antibody were analyzed by immunoblotting (IB) using anti-Abl and anti-PKCδ antibodies at the indicated time points. Shown are representative data of two independent experiments. (C) ER fractions were isolated from the penumbra area of rats brains that had been subjected to 2 hours MCAO followed by 24 hours of reperfusion. Immunoprecipitates obtained using anti-Abl antibody were analyzed by immunoblotting with anti-PKCδ and anti-Abl antibodies. Data are representative of results obtained from five rats per group.

Abl is required for PKCδ kinase activity in the ER following ER stress

Immunoprecipitation-based kinase assays were used to evaluate the activity of individual PKC isozymes following cell stimulation (Disatnik et al., 2002). As shown in Fig. 4E, in ER fractions, 30 minutes of Tm treatment significantly increased levels of phosphorylated histone (*P*-histone) by the immunoprecipitated PKCδ, and δV1-1 treatment completely blocked this increase. Importantly, the Tm-induced increase in the catalytic activity of PKCδ – as measured by histone phosphorylation – was abolished in cells in which Abl was knocked down. This result suggests that Abl is crucial for PKCδ kinase activity under ER stress.

Abl is required for tyrosine phosphorylation of PKCδ in the ER following ER stress

Abl is a tyrosine kinase (Shaul, 2000), and tyrosine phosphorylation of PKCδ represents an early event in the apoptotic pathways and plays an important role in the pro-apoptotic effects of PKCδ in response to various stimuli, including H_2O_2, UV radiation, ceramide and ionizing irradiation (Brodie and Blumberg, 2003). We therefore determined whether the interaction of Abl and PKCδ is mediated by tyrosine phosphorylation in response to ER stress. Immunoblot analysis of PKCδ immunoprecipitated with antibody against phosphorylated tyrosine revealed increased tyrosine phosphorylation of PKCδ in the Tm-treated group in the ER-enriched fractions following 30 minutes of Tm treatment, compared with control groups (Fig. 4F). δV1-1 significantly inhibited the rise in tyrosine phosphorylation of PKCδ (Fig. 4F), and tyrosine phosphorylation of PKCδ in the ER fractions was completely abrogated in cells in which Abl was knocked down. Abl phosphorylation by PKCδ is conflicting. Here, we did not find PKCδ-dependent tyrosine or serine/threonine phosphorylation of Abl (data not shown). These results indicate that, directly or indirectly, Abl is responsible for tyrosine phosphorylation of PKCδ in the ER. Furthermore, the findings support the possibility that binding of PKCδ to Abl precedes its translocation to the mitochondria.

Interaction of PKCδ and Abl is essential for ER-stress-induced cell death

To assess the functional significance of the interaction between Abl and PKCδ, we next examined the role of PKCδ and Abl in ER-stress-induced apoptosis. First, we found that silencing either Abl or PKCδ reduced ER-stress-mediated activation of JNK (JNK phosphorylation) (Fig. 5A), whereas little effect on CHOP induction and caspase-12 activation was observed (data not shown). These data suggest that both PKCδ and Abl participate in ER-stress-mediated apoptosis through JNK activation. JNK has a number of targets, including Bad and Bax, which mediate the collapse of mitochondrial membrane potential, leading to apoptosis (Weston and Davis, 2007). As shown in Fig. 5B, silencing of either Abl or PKCδ completely blocked the increase in mitochondrial-membrane-associated Bax (the activated form of the protein) in response to Tm treatment. Second, treatment of cells with siRNA targeting PKCδ or Abl completely abolished apoptosis, as measured by TUNEL staining (Fig. 5C). These data suggest that mediation of ER-stress-induced apoptosis by PKCδ and Abl is dependent on activation of the JNK signaling cascade.

Finally, we determined whether PKCδ catalytic activity is required for the translocation of PKCδ-Abl from the ER to the mitochondria. Neuo2a cells were first treated with Tm to cause interaction of PKCδ with Abl in the ER. After 30 minutes, the time

required for the PKCδ-Abl complex to form (Fig. 3), the cells were treated with rottlerin, which inhibits the catalytic activity of PKCδ (Gschwendt et al., 1994). Tm treatment enables the interaction of PKCδ-Abl in the ER but, if translocation from the ER to the mitochondria depends on PKCδ catalytic activity, rottlerin would block the movement of the PKCδ-Abl complex to the mitochondria. We found that rottlerin completely inhibited PKCδ-Abl translocation to the mitochondria 3 hours after Tm treatment. In fact, levels of the PKCδ-Abl complex in ER fractions were higher under these conditions (Fig. 6A). These data strongly support our hypothesis that PKCδ-Abl indeed translocates from the ER to mitochondria in response to ER stress, and that the catalytic kinase activity of PKCδ is crucial for this translocation.

We next determined the effect on ER-stress-mediated apoptosis in Neuro2a cells when formation of the PKCδ-Abl complex is inhibited. As shown in Fig. 6B, treatment with rottlerin 30 minutes after treatment with Tm greatly decreased the levels of Bax in the mitochondrial fraction as well as it decreased the release of cytochrome *c*, and significantly reduced the number of TUNEL-positive cells (Fig. 6C). These data demonstrate that translocation

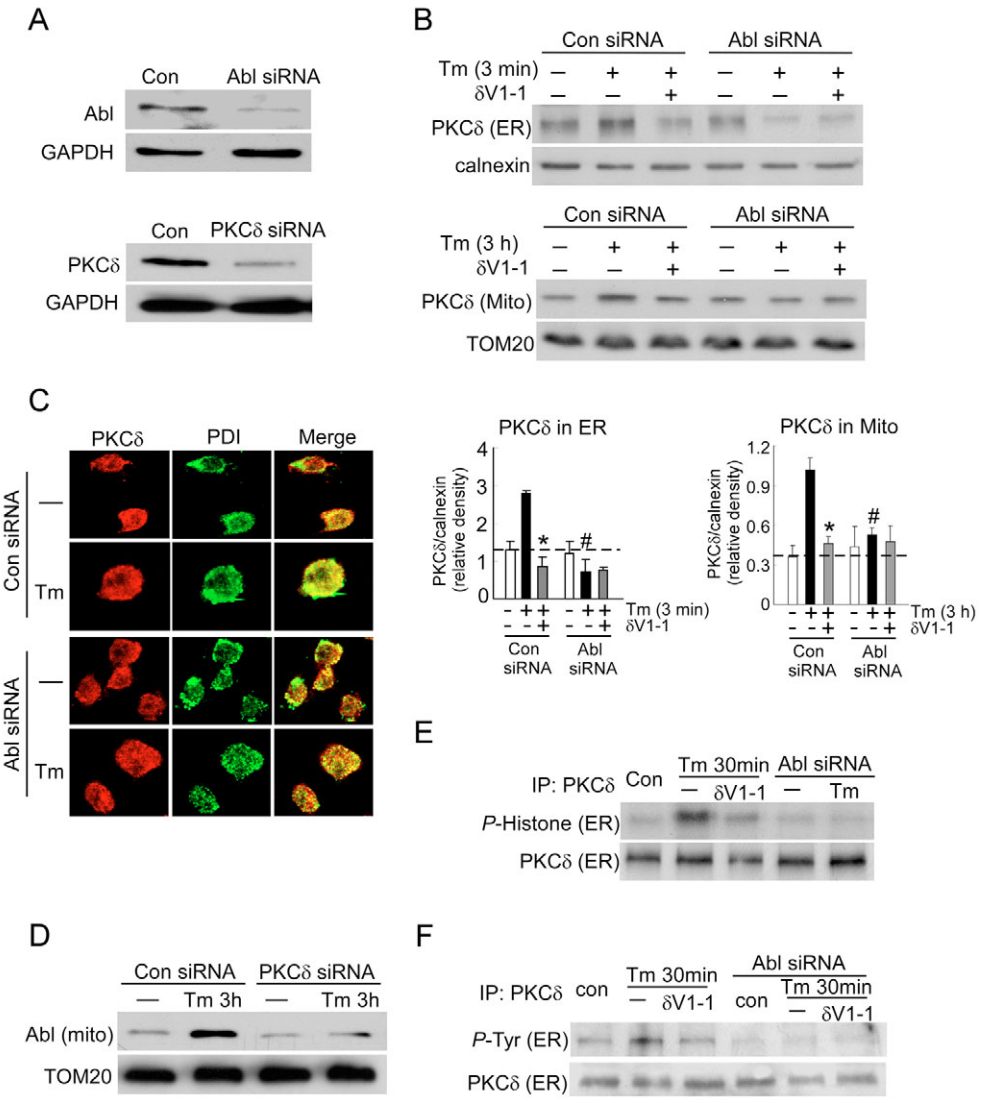

Fig. 4. PKCδ and Abl are both required for the response to ER stress. Neuro2a cells were transfected with control siRNA, Abl siRNA or PKCδ siRNA. After 48 hours, cells were pretreated with δV1-1 (1 μM) for 15 minutes followed by Tm (5 μg/ml) treatment. (A) Total-cell lysates were analyzed by western blotting to confirm knockdown of Abl (upper panel) and PKCδ (lower panel). GAPDH in total lysates was used as internal loading control. (B) (Top two panels) Levels of PKCδ were analyzed by western blotting in the ER (upper panel) and mitochondrial (middle panel) fractions at the indicated time. (Lower panel) Histogram depicting the amount of PKCδ associated with the ER or mitochondria in Neuro2a cells. Data are expressed as the mean ± s.e. of three independent experiments. $^{*}P<0.05$ vs Tm treatment, $^{\#}P<0.05$ vs Tm treatment in cells transfected with control siRNA. (C) Representative confocal images of PKCδ (red) and PDI (green) following 3 minutes of Tm treatment in the cells transfected with control or Abl siRNA. The data are from three independent experiments. Original magnification was ×60. (D) Levels of Abl in mitochondrial fractions were analyzed by western blot after 3 hours of Tm treatment in cells transfected with control siRNA or PKCδ siRNA. Shown are representative data of three independent experiments. (E) Cell lysates from the ER fractions were subjected to immunoprecipitation (IP) with anti-PKCδ. In vitro kinase assays were carried out with or without δV1-1 or knockdown of Abl using histone as a substrate. Data are representative of two independent experiments. (F) Cell lysates from the ER fractions were subjected to immunoprecipitation (IP) using anti-PKCδ antibody. The immunoprecipitates were analyzed by immunoblotting (IB) using anti-*P*-Tyr and anti-PKCδ antibodies. Data are representative for three independent experiments.

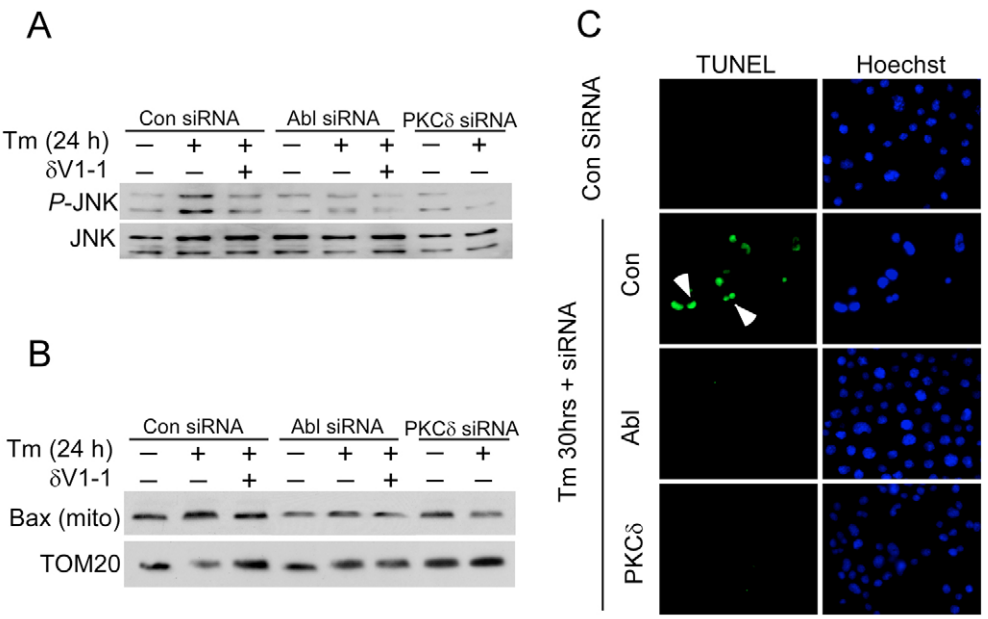

Fig. 5. PKCδ and Abl mediate ER-stress-induced mitochondria-dependent apoptosis. Neuro2a cells were transfected with control siRNA, or siRNA targeting Abl or PKCδ. After 48 hours, cells were pretreated with δV1-1 (15 minutes, 1 μM) followed by treatment with Tm (5 μg/ml). (A) After 24 hours of Tm treatment, total cell lysates were subjected to western blotting with the indicated antibodies. Data are representative results of three independent experiments. (B) Mitochondrial fractions were isolated from Neuro2a cells treated with Tm for 24 hours after transfection, and subjected to western blot with anti-Bax antibody. TOM20 was used as loading control. Shown are from three independent experiments. (C) TUNEL assay. Neuro2a cells were treated with Tm for 30 hours before staining with TUNEL. Representative data are from two independent experiments. Original magnification was ×40.

of PKCδ-Abl to the mitochondria is essential for ER-stress-induced mitochondrium-dependent apoptosis.

Discussion

In this study, we have identified three new steps in ER-stress-induced apoptosis. (1) PKCδ translocates to the ER in a process that is dependent on Abl, and a PKCδ-Abl complex is formed; (2) the PKCδ-Abl complex translocates to mitochondria in a process that is dependent on PKCδ tyrosine phosphorylation and PKCδ catalytic activity; (3) PKCδ-Abl complex formation activates JNK-induced mitochondrial-dependent apoptosis.

Step 1 – formation of the PKCδ-Abl complex following ER stress

Using western blot analysis, immunofluoresence and electron microscopy, we demonstrated that, shortly after induction of ER stress, PKCδ translocates to the ER in vitro and in vivo. Here, we provided evidence that Abl is required for PKCδ translocation to the ER; silencing of Abl inhibits this translocation. We also found that PKCδ and Abl interact in the ER in response to ER stress, as evidenced by co-immunoprecipitation studies. Finally, Abl-dependent tyrosine phosphorylation of PKCδ occurs in the ER in response to ER stress. These findings indicate that translocation of PKCδ to the ER and its binding to Abl are early events in the ER-stress response. In addition, PKCδ translocation to the nucleus has been reported to be important for DNA-damage-induced apoptosis (Basu, 2003). Therefore, PKCδ may regulate the induction of some genes in response to ER stress, for example that of the transcription factor CHOP, whose expression is induced upon ER stress.

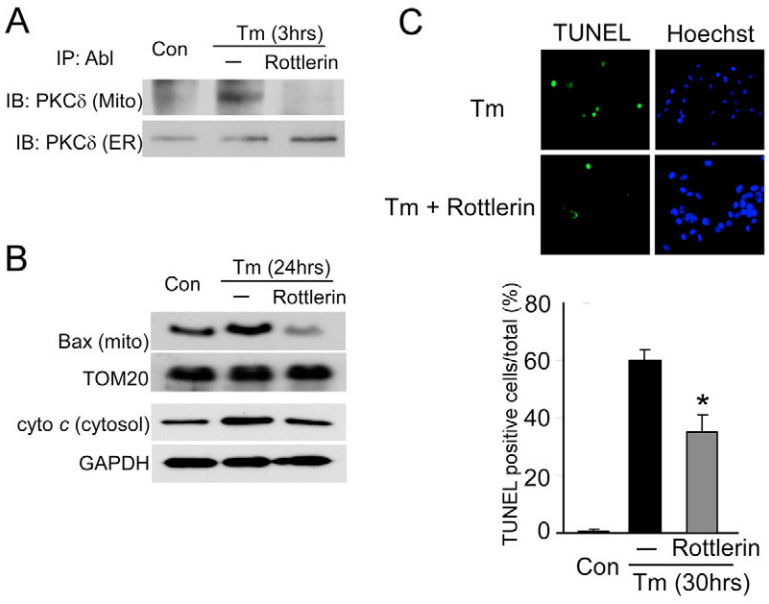

Fig. 6. Disassociation of the PKCδ-Abl complex by rottlerin reduces ER-stress-induced mitochondrial-dependent apoptosis. Neuro2a cells were treated with Tm (5 μg/ml). After 30 minutes, cells were treated with rottlerin (5 μM). (A) ER and mitochondrial fractions were isolated after a total of 3 hours of Tm treatment. Immunoprecipitates obtained by using anti-Abl antibodies were analyzed by immunoblotting using anti-PKCδantibody. Representative data are from three independent experiments. (B) After 24 hours of Tm treatment, mitochondrial and cytosolic fractions were subjected to western blotting using anti-Bax and anti-cytochrome *c* antibodies, respectively. Tom20 and GAPDH antibodies were used as loading controls for each fraction. Data are representative for two independent experiments. (C) Neuro2a cells were treated with Tm for 30 hours before staining with TUNEL. (Top) Representative data of three independent experiments. Original magnification was ×40. (Bottom) TUNEL-positive cells are expressed as a percentage of the number of total cells, as determined by staining after fixation with Hoechst dye. *P<0.05 vs Tm treatment.

Step 2 – translocation of PKCδ-Abl to mitochondria following ER stress

As discussed earlier, a connection between PKCδ and Abl has been previously described (Sun et al., 2000; Yuan et al., 1998). After exposure to hydrogen peroxide, cells overexpressing the regulatory domain of PKCδ exhibit a lower activity of Abl (Sun et al., 2000). Moreover, a PKCδ-dependent increase in the activity of Abl was reported in cells treated with a combination of cisplatin and methylglyoxal (Godbout et al., 2002). These studies suggest a role for PKCδ in Abl activity. In our study, we found that ER stress induced a Abl-mediated increase in the catalytic activity of PKCδ in the ER in response to ER stress. Moreover, inhibition of PKCδ translocation by δV1-1 inhibits formation of the PKCδ-Abl complex, and inhibition of PKCδ catalytic activity by rottlerin prevents translocation of the complex from the ER to the mitochondria. In this study, we were unable to determine whether Abl and PKCδ interaction is mediated by direct contact or whether the two proteins are recruited to a larger complex through interaction with other proteins. Also, because rotterlin has been reported to inhibit a number of PKCδ-independent pathways and targets (Bazuine et al., 2004; Leitges et al., 2001; Soltoff, 2007), our conclusion regarding the role of PKCδ in the process is mainly based on the results obtained from our experiments using δV1-1 (Chen et al., 2001) or siRNA. Together, our findings indicate that the activities of PKCδ and Abl are intricately linked in response to ER stress, and that translocation of Abl from the ER to the mitochondria reflects the translocation of PKCδ-Abl to this compartment.

Step 3 – formation of the PKCδ-Abl complex is required to communicate ER stress to the mitochondria, triggering JNK-dependent ER-stress-induced apoptosis

Accumulating evidence suggest that the mitochondria are important components of ER-stress-induced apoptotic events (Breckenridge et al., 2003). Following ER stress, phosphorylated JNK interacts with the intrinsic cell-death pathway at several points; it has been shown to be both sufficient and necessary for translocation of Bax and Bad to mitochondria, and the release of cytochrome *c* from the mitochondria (Weston and Davis, 2007). Moreover, inhibition of JNK, either by a specific inhibitor or by decreasing its level using siRNA, inhibits ER-stress-induced apoptosis (Joo et al., 2007; Kerkela et al., 2006). These data collectively suggest that PKCδ-induced JNK activation is crucial for the ER-stress-induced cell-death cascade. Notably, our study demonstrates that either knockdown of PKCδ or treatment with δV1-1 inhibits ER-stress-induced JNK phosphorylation, Bax accumulation in the mitochondria and the resulting apoptosis. Consistent with the finding that Abl-deficient mouse embryo fibroblasts are protected from ER stress (Ito et al., 2001), we also found that knockdown of Abl increased the resistance of Neuro2a cells to ER stress through the same signaling pathway as PKCδ. These data suggest that PKCδ and Abl together trigger JNK-dependent ER-stress-induced apoptosis. However, how the complex affects JNK signaling is not known yet. In addition, we noticed a lag time between PKCδ and Abl translocation and JNK activation, suggesting that JNK may be activated via unknown downstream enzyme mediators.

ER stress produces reactive oxygen species (ROS) that further worsen ER function and, consequently, cause neuronal cell death (Gorlach et al., 2006). Moreover, exposing primary neuronal cell cultures to an NO-donor results in the inactivation of the ER Ca^{2+} pump, depletion of ER Ca^{2+} stores and a long-lasting suppression of protein synthesis (Doutheil et al., 2000). These studies suggest a crucial role for oxidative stress on ER functions. PKCδ is activated by oxidative stress (Kanthasamy et al., 2003; Nitti et al., 2005) and inhibition of PKCδ diminishes oxidant-induced cell death (Choi et al., 2006; Hu et al., 2007). Thus, it is possible that, upon ER stress, PKCδ is activated through production of ROS by ER stress. In addition, we found that localization of Abl to the ER is essential for PKCδ translocation to the ER. Thus, it is possible that some proteins in the ER – such as Abl – sense the ER stress, subsequently recruit PKCδ from the cytosol, thereby leading to its activation.

We have noticed that δV1-1 treatment was more effective regarding ER dysfunction in the in vivo stroke model than in the in vitro culture, inhibiting all of the three ER-stress apoptotic mediators (CHOP, caspase-12 and JNK). By contrast, following Tm or Tg treatment only phosphorylation of JNK was inhibited by δV1-1 in culture (Fig. 2 and supplementary material Fig. S1). These differences between the in-vitro and in-vivo models may be due to the strength as well as the timing of the ER stress stimuli. Nevertheless, our findings provide support for a model in which ER stress triggers formation of the PKCδ-Abl complex in the ER and translocation of the complex to the mitochondria amplifies apoptotic signals via activation of the mitochondrial apoptotic pathway (Fig. 7).

Involvement of PKCδ-Abl in ER stress in response to stroke

Suppression of the ER-stress-mediated apoptotic signaling cascade following cerebral ischemia by either pharmacological intervention or knockdown of ER-stress-related apoptotic mediator, such as CHOP or JNK, reduce neuronal cell death (Qi et al., 2004a; Qi et

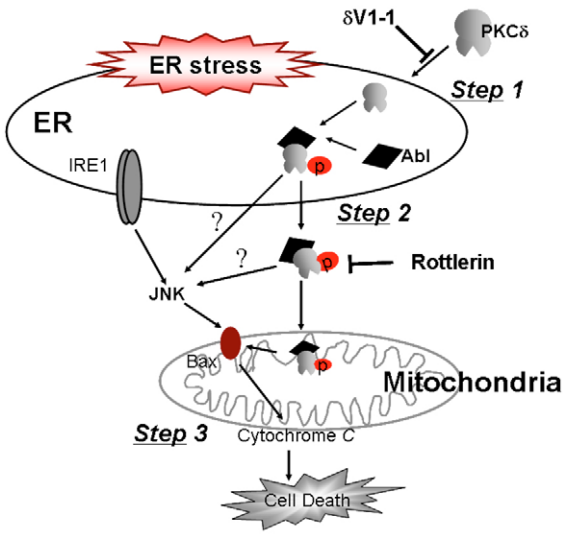

Fig. 7. Steps in ER-stress-induced apoptosis and communication of this signaling event from the ER to the mitochondria by the PKCδ-Abl complex. Several steps in the transduction of ER stress signaling leading to apoptosis have been identified. (Step 1) ER stress triggers translocation of PKCδ to the ER where it binds to Abl and is (directly or indirectly) phosphorylatedon tyrosine residues by Abl. (Step 2) The PKCδ-Abl complex translocates to mitochondria in a process that depends on PKCδ catalytic activity. Abl is required for PKCδ translocation to the mitochondria and, vice versa, PKCδ is required for Abl translocation to this organelle. (Step 3) PKCδ-Abl complex activates JNK-mediated mitochondrium-dependent apoptotic cascade. The PKCδ-specific peptide inhibitor δV1-1, inhibits PKCδ translocation to the ER and the subsequent interaction of PKCδ and Abl, thereby suppressing ER-stress-mediated apoptosis. Rottlerin, an inhibitor of PKCδ catalytic activity, blocks the translocation of PKCδ-Abl complex to mitochondria and, thus, inhibits ER-stress-induced mitochondrium-dependent apoptosis.

al., 2004b; Tajiri et al., 2004), indicating that ER-stress response is a potential therapeutic target in cerebral ischemia. Consistent with a previous study (Li et al., 2005), we found here that 2 hours of ischemia followed by 24 hours reperfusion in a rat stroke model triggers CHOP induction, caspase-12 activation and JNK phosphorylation (Fig. 2 and supplementary material Fig. S1). Moreover, activation of these signals was found in the penumbra area of the ipsilateral hemisphere, and all were blocked by the PKCδ inhibitor δV1-1.

Our finding of a significant association between PKCδ and Abl in the penumbra area of rat brains after stroke suggests that the PKCδ-Abl complex contributes to the response pathogenesis of this disease through its role in the ER-stress response. We previously reported that delivery of δV1-1 – even 6 hours after reperfusion – is still protective in the same model (Bright et al., 2004). Our current finding that inhibition of δPKC by δV1-1 protects against the ER-stress-mediated apoptotic event may explain, at least in part, the mechanism by which δPKC mediates the late cellular response to stroke. Taken together, our present findings represent a potential mechanism by which cells modulate apoptosis in response to ER stress, and suggest that a PKCδ inhibitor is a possible therapeutic agent for diseases in which ER stress contributes to the pathology.

Materials and Methods

Materials

Tunicamycin, thapsigargin, protease inhibitor cocktail and phosphatase inhibitor cocktails were purchased from Sigma-Aldrich. Rottlerin was from CalBioChem (CA). Antibodies directed against PKCδ, CHOP (also known as DDIT3 or GADD153), calnexin, Abl and TOM20 were purchased from Santa Cruz Biotechnology. Antibodies against phosphorylated JNK, JNK and Bax were purchased from Cell Signaling Biotechnology. Antibody against phosphorylated tyrosine was from Upstate Biotechnology. Cytochrome *c* was from MitoSciences (Eugene, OR). Anti-mouse IgG and anti-rabbit IgG, peroxidase-linked species-specific antibodies were from Amersham Biosciences. Anti-GAPDH antibody clone 6C5 was from Advanced Immunochemical. The PKCδ-specific antagonist peptide δV1-1 [PKCδ inhibitor, amino acids 8-17 (SFNSYELGSL)] were synthesized by American peptides and conjugated to a TAT carrier peptide, amino acids 47–57 (YGRKKRRQRRR) via a cysteine-cysteine bond at their N-termini, as previously described (Chen et al., 2001).

Cell culture

Mouse neuroblastoma Neuro2a cells were maintained in modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. All cultured cells were maintained at 37°C in 5% CO_2 in 95% air.

Apoptosis assay

Neuro2a cells were treated with δV1-1 (1 μM) for 15 minutes before Tm or Tg treatments. After 30 hours, apoptotic cells were identified using the TUNEL technique per the manufacturer's instructions (in situ cell death detection kit, Roche Applied Science). Labeled Neuro2a cells were analyzed with a fluorescent microscope.

Middle cerebral artery occlusion stroke model

Transient stroke was induced in adult male Sprague Dawley rats (250-280 g) using an occluding intraluminal suture, as described previously (Bright et al., 2004). Briefly, an uncoated 30-mm-long segment of 3-0 nylon monofilament suture with the tip rounded by a flame was inserted into the stump of the external carotid artery and advanced into the internal carotid artery ~19-20 mm from the bifurcation to occlude the ostium of the middle cerebral artery (MCA). At the end of the ischemic period (2 hours), the suture was removed and the animal was allowed to recover. Animals were maintained under isoflurane anesthesia during all surgical procedures. Tat carrier or δV1-1 peptides were delivered as an intraperitoneal dose (0.2 mg/Kg). Physiological parameters including body temperature (35-38°C) and respiration rate were monitored and maintained using a heat blanket and anesthetic adjustment.

ER and mitochondria isolation

Neuro2a cells were washed with cold phosphate-buffered saline (PBS) and incubated on ice in lysis buffer A (250 mM sucrose, 20 mmol/l HEPES-NaOH pH 7.5, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1:300 protease inhibitor cocktail, 1:300 phosphatase inhibitor cocktail) for 30 minutes. Cells were then scraped off the dish, disrupted by repeated aspiration through a 25-gauge needle. Brain tissue was minced and ground using a pestle in lysis buffer. The homogenates were spun at 800 ***g*** for 10 minutes at 4°C and the resulting supernatants were spun at 10,000 ***g*** for 20 minutes at 4°C. The pellets were suspended in lysis buffer containing 1% Triton X-100 and formed mitochondria-rich fractions. The new supernatants were spun at 100,000 ***g*** for 1 hour at 4°C and the pellet, corresponding to the ER-enriched fraction, was resuspended in lysis buffer containing 1% Triton X-100.

Preparation of total-cell lysate

Samples were processed in the following lysis buffer B (10 mmol/l HEPES-NaOH pH 7.5, 150 mmol/l NaCl, 1 mM EGTA, 1% Triton X-100, 1:300 protease inhibitor cocktail, 1:300 phosphatase inhibitor cocktail). After 20 minutes of incubation on ice, homogenates were spun in an Eppendorf 5415C centrifuge at 14,000 rpm for 20 minutes at 4°C. The supernatants correspond to the total-cell lysates.

Western blot analysis

Protein concentrations were determined using the Bradford assay. Ten micrograms of protein was resuspended in Laemmli buffer, loaded on SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibody followed by visualization by ECL.

Electron microscopy

ER fractions were isolated from tissue of brains subjected to 2 hours ischemia followed by 4 hours of reperfusion. The specimens were fixed in 2% formaldehyde and 0.5% glutaraldehyde for 40 minutes at 4°C in 0.1 mol/l PBS pH 7.2, and then rinsed twice in PBS buffer for 10 minutes each. The fixed samples were dehydrated in an ascending ethanol series up to 95% ethanol and infiltrated in a 1:1 mixture of LR White and ethanol for 1-2 hours under rotation. This was followed with a 1:2 mixture of LR White:ethanol and finally with pure LR White. Samples were embedded into gelatin capsules and cured at 55°C in an oven overnight. The sections were cut with an LKB V ultratome and collected on formvar-coated nickel grids. The grids were incubated for 10 minutes at room temperature with PBS buffer for rehydration and then treated with 1% normal goat serum (NGS) for 1 hour to block non-specific reactions. The sections were incubated at room temperature with primary antibody against PKCδ (1:25) for 1 hour, washed with PBS and incubated for 1 hour with goat-anti-rabbit serum coupled to 10-nm gold particles. After washes with PBS, grids were stained with 2% uranyl acetate for 5 minutes and examined with a CM12 Phillips microscope.

Confocal immunofluorescence microscopy

Neuro2a cells cultured on eight-well glass chambers were washed with cold PBS, fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% normal goat serum and incubated overnight at 4°C with a rabbit antibody against PKCδ (1:100) and a monoclonal antibody against PDI (1:1000) from the ER labeling kit (Invitrogen). Sections were washed with PBS and incubated for 60 minutes with TRITC-labeled goat anti-rabbit antibody and Alexa-Fluor-488-labeled goat anti-mouse antibody followed by incubation with Hoechst 33342 dye (1:10,000) for 15 minutes. Mitochondria were stained with 0.05 ng of Mitotracker Green FM (Molecular Probes) per slide. Coverslips were mounted and slides were imaged by microscopy (BioRad Radiance 2100).

Immunoprecipitation

Solubilized proteins (200-500 μg) in lysis buffer A containing 1% Triton X-100 were incubated with primary antibody against Abl (2μg/ml) or PKCδ (2μg/ml) at 4°C for 3 hours and precipitated with protein-A–sepharose (Santa Cruz Biotechnology) at 4°C for 1 hour. After centrifugation, the pellets were washed with lysis buffer four times. The immunoprecipitates dissolved in SDS-sample buffer were analyzed by western blotting.

RNA interference

Small interfering RNA (siRNA) duplexes targeting Abl and PKCδ were obtained from Santa Cruz Biotechnology. Adhered Neuro2a cells at 50% confluency were transfected with siRNA targeting Abl or PKCδ or with control siRNA using a transfectional kit (Gentaris, CA) according to manufacturer's instructions. The experiments were performed 48 hours after transfection.

Kinase assay

Solubilized proteins (200 μg) in lysis buffer A containing 1% Triton X-100 were immunoprecipitated using primary antibody against PKCδ (2μg/ml) at 4°C for 2 hours and precipitated with protein-A–sepharose (Santa Cruz Biotechnology) at 4°C for 1 hour. Immunocomplexes were washed three times with lysis buffer and once with binding buffer (20 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 25 μM ATP). PKCδ activity of immunoprecipitated fractions was assayed by adding 40 μl of binding buffer containing 5 μCi [γ-^{32} P]ATP (5000 Ci/mmole, Amersham) and 40 μg histone III-S (Sigma). After a 25-minute incubations at 37°C, assays were terminated by adding sample buffer. The samples were loaded on a 10% SDS acrylamide gel, and the levels of phosphorylated histone were quantified using autoradiograpy.

Statistics

Data are expressed as the mean ± s.e. Unpaired Student's *t*-test for differences between two groups, 1-factor ANOVA with Fisher's test for differences among >2 groups, and Fisher's test for categorical data were used to assess significance ($P<0.05$).

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