

Bax inhibitor 1 regulates ER-stress-induced ROS accumulation through the regulation of cytochrome P450 2E1

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Summary

This study investigated the molecular mechanism by which Bax inhibitor 1 (BI1) abrogates the accumulation of reactive oxygen species (ROS) in the endoplasmic reticulum (ER). Electron uncoupling between NADPH-dependent cytochrome P450 reductase (NPR) and cytochrome P450 2E1 (P450 2E1) is a major source of ROS on the ER membrane. ER stress produced ROS accumulation and lipid peroxidation of the ER membrane, but BI1 reduced this accumulation. Under ER stress, expression of P450 2E1 in control cells was upregulated more than in BI1-overexpressing cells. In control cells, inhibiting P450 2E1 through chemical or siRNA approaches suppressed ROS accumulation, ER membrane lipid peroxidation and the resultant cell death after ER stress. However, it had little effect in BI1-overexpressing cells. In addition, BI1 knock down also increased ROS accumulation and expression of P450 2E1. In a reconstituted phospholipid membrane containing purified BI1,

NPR and P450 2E1, BI1 dose-dependently decreased the production of ROS. BI1 bound to NPR with higher affinity than P450 2E1. Furthermore, BI1 overexpression reduced the interaction of NPR and P450 2E1, and decreased the catalytic activity of P450 2E1, suggesting that the flow of electrons from NPR to P450 2E1 can be modulated by BI1. In summary, BI1 reduces the accumulation of ROS and the resultant cell death through regulating P450 2E1.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/122/8/1126/DC1>

Key words: BAX inhibitor 1, Reactive oxygen species, Endoplasmic reticulum, NADPH-P450 reductase, Cytochrome P450, Nicotinamide adenine dinucleotide phosphate, Microsomal monooxygenase, Unfolded protein response

Introduction

The anti-apoptotic protein, BI1 (Bax inhibitor 1) (Kadowaki et al., 2005), protects against apoptosis induced by ER stress (Xu and Reed, 1998). Cells isolated from *BI1*^{-/-} mice exhibit hypersensitivity to apoptosis induced by ER stress (Chae et al., 2004). In *BI1*^{-/-} mice, the ischemia/reperfusion-induced unfolded protein response is significantly increased, leading to increased cell death (Bailly-Maitre et al., 2006).

BI1 is protective, in part, via its pH-sensitive regulation of intra-ER Ca²⁺ levels (Kim et al., 2008). BI1 also regulates ROS production (Kawai-Yamada et al., 2004; Baek et al., 2004) in the ER by modifying heme oxygenase 1 (HO1) expression.

In the ER, the major source of reactive oxygen species (ROS) is the microsomal monooxygenase (MMO) system, which is composed of cytochrome P450 (P450), NADPH-P450 reductase (NPR) and phospholipids (Premereur et al., 1986; Davydov, 2001). Although the main function of MMO is mixed-function oxygenation of exogenous compounds (xenobiotics) and some endogenous substrates, the MMO system also leads to the release of large amounts of reactive oxygen species (ROS), such as superoxide anion radical and H₂O₂, from the P450 enzyme (especially cytochrome P450 2E1) without substrate oxidation (Nieto et al., 2002). The efficiency, or degree of

coupling, of electron transfer from NADPH to P450 is usually less than 50-60%, often being as low as 0.5-3.0%. This 'electron leakage' contributes significantly to ROS production during redox cycling between NPR and eukaryotic P450s.

In this study, we hypothesized that ER stress results in the accumulation of ROS generated from the MMO system and that BI1 can affect this system, leading to regulation of ROS on the ER membrane.

Results

BI1 regulates ER stress-associated ROS accumulation and ER membrane lipid peroxidation

To examine the specific mechanism of ROS regulation by BI1, we first generated a stable monoclonal HepG2/BI1 clone (Fig. 1A, inset) and measured ROS levels in Neo (Neo-resistant vector-transfected: control) and BI1 (BI1-overexpressing) cells after treatment with the ER-stress inducers thapsigargin or tunicamycin. Treatment with thapsigargin significantly increased ROS levels in Neo cells, but not in BI1 cells (Fig. 1A). BI1 also inhibited tunicamycin-induced ROS accumulation. In addition, ER stress induced lipid peroxidation on the ER membrane in Neo cells, whereas BI1 cells showed smaller changes in the amount of reacted malondialdehyde (MDA) (Fig. 1B).

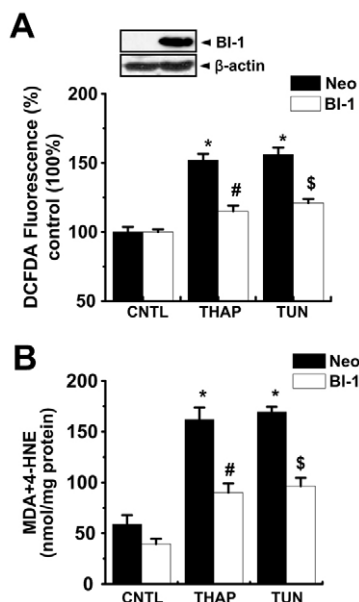


Fig. 1. Effect of BI1 on ER stress-initiated ROS accumulation. (A) Neo- (neomycin-resistant) and BI1-overexpressing HepG2 cells (namely Neo and BI1) were lysed, and the lysates were then immunoblotted with anti-HA (inset). The Neo and BI1 cells were treated with 5 μ M thapsigargin or 5 μ g/ml tunicamycin for 20 hours. DCFDA was loaded into the cells, and the fluorescence was measured. (B) Neo and BI1 cells were treated with either 5 μ M thapsigargin or 5 μ g/ml tunicamycin for 20 hours. The microsomal fractions were then isolated from the Neo and BI1 cells, and the ER lipid peroxidation was measured. The data indicate the mean \pm s.e.m. ($n=4$). * $P<0.05$, significantly different from control-Neo cells; # $P<0.05$, significantly different from 5 μ M thapsigargin-treated Neo cells; \$ $P<0.05$, significantly different from 5 μ g/ml tunicamycin-treated Neo cells. CNTL, control; THAP, thapsigargin; TUN, tunicamycin.

ER stress-induced ROS and cell death are associated with P450 2E1, which is regulated by BI1

P450 2E1 is associated with increased ROS (Nieto et al., 2002), and BI1 cells showed decreased basal expression of P450 2E1 (Fig. 2A). Western blot experiments showed that thapsigargin and tunicamycin increased the expression of P450 2E1 in Neo cells, whereas BI1 cells showed no induction of P450 2E1 (Fig. 2B).

To determine whether ER stress-induced accumulation of ROS occurs upstream or downstream of P450 2E1 induction, the expression of P450 2E1 was monitored by immunoblotting of cells treated with the antioxidant N-acetylcysteine (NAC) or with reduced glutathione (GSH). The expression of P450 2E1 was not affected by either NAC or GSH (supplementary material Fig. S1A,B). These results suggest that P450 2E1 induction is followed by ROS production in ER stress-exposed cells.

The effect of P450 2E1 was also confirmed in a BI1 knockdown system. BI1 siRNA-transfected cells reversed BI1-induced p450 2E1 inhibition, showing increased expression of P450 2E1 (supplementary material Fig. S2A). ROS accumulation was also modestly increased in the BI1 knockdown system (supplementary material Fig. S2B). Thus, BI1 regulates P450 2E1 induction and the resultant accumulation of ROS.

We next tested whether 4-methylpyrazole (4-MP), a specific P450 2E1 inhibitor (Gong et al., 2003; Halpert et al., 1994), could block lipid peroxidation on the ER membrane. 4-MP blocked thapsigargin- or tunicamycin-stimulated lipid peroxidation in Neo cells (Fig. 3A). Similarly, inhibition of P450 2E1 decreased the amount of H_2O_2 in

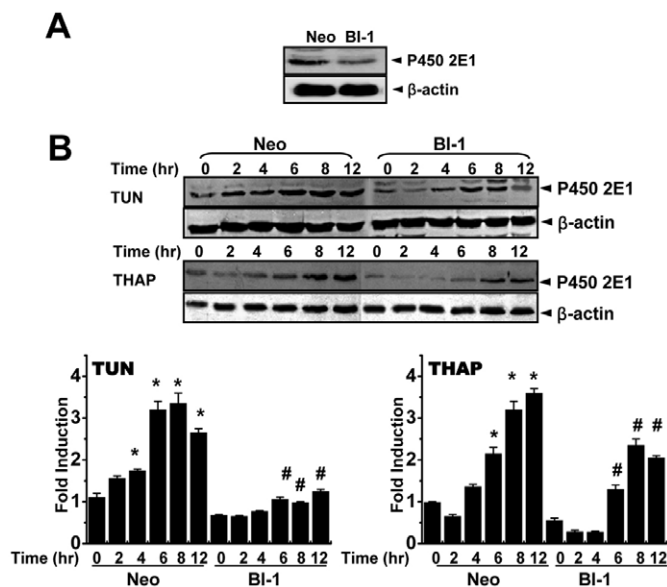


Fig. 2. Effect of BI1 on the expression of P450 2E1. (A) Neo and BI1 cells were immunoblotted with anti-P450 2E1 and β -actin antibodies. (B) Neo and BI1 cells were treated with 5 μ M thapsigargin or 5 μ g/ml tunicamycin for 0, 2, 4, 6, 8 and 12 hours. Cell lysates were immunoblotted with anti-P450 2E1 and β -actin antibodies. The expression of P450 2E1 was normalized to that of β -actin as a relative ratio. The data indicate the mean \pm s.e.m. ($n=7$). * $P<0.05$, significantly different from the amount of P450 2E1 expressed in the Neo cells without any treatment; # $P<0.05$, significantly different from the amount of P450 2E1 expressed in the Neo cells under each of the indicated conditions.

ER-stressed Neo cells (Fig. 3B). In ER stress-exposed BI1 cells, 4-MP additionally inhibited the reduced level of ROS and lipid peroxidation (Fig. 3A,B). To modulate P450 2E1 levels directly, we used a siRNA directed against P450 2E1 and decreased the expression of P450 2E1 without affecting the expression of BI1 (Fig. 3C). The decreased P450 2E1 expression significantly inhibited ER stress-induced ROS accumulation (Fig. 3C), and tended to augment the decreased ROS levels in the ER stress-exposed BI1 cells. The effects on ROS by chemical and gene silencing approaches in BI1 cells (Fig. 3A-C) are consistent with the finding that P450 2E1 induction is not completely blocked by BI1 (Fig. 1A).

To extrapolate the role of P450 2E1 on ER stress-initiated ROS accumulation to cell death, we measured the viability of Neo and BI1 cells that were exposed to ER stress in the presence of 4-MP or under P450 2E1 knock-down. ER stress-induced cell death could be regulated by both of approaches-chemical and siRNA (Fig. 4A,B). In particular, 4-MP dose-dependently inhibited ER stress-induced cell death at concentrations (1 and 10 mM), consistent with an effect on P450 2E1 activity (Amato et al., 1998; Haorah et al., 2005). P450 2E1 siRNA also significantly inhibited cell death, but less than 4-MP treatment, probably owing to limitations of silencing efficiency. N-acetylcysteine (NAC) or reduced glutathione (GSH) treatment of cells exposed to thapsigargin or tunicamycin blocked cell death in Neo cells, but did not dramatically affect BI1 cells (Fig. 4C), as reported in another cell system, HT1080 cells (Lee et al., 2007).

BI1 decreases the production of ROS by P450 2E1 in a reconstitution system

To study the function of BI1, we purified and identified recombinant BI1 protein (supplementary material Fig. S3A,B), and measured

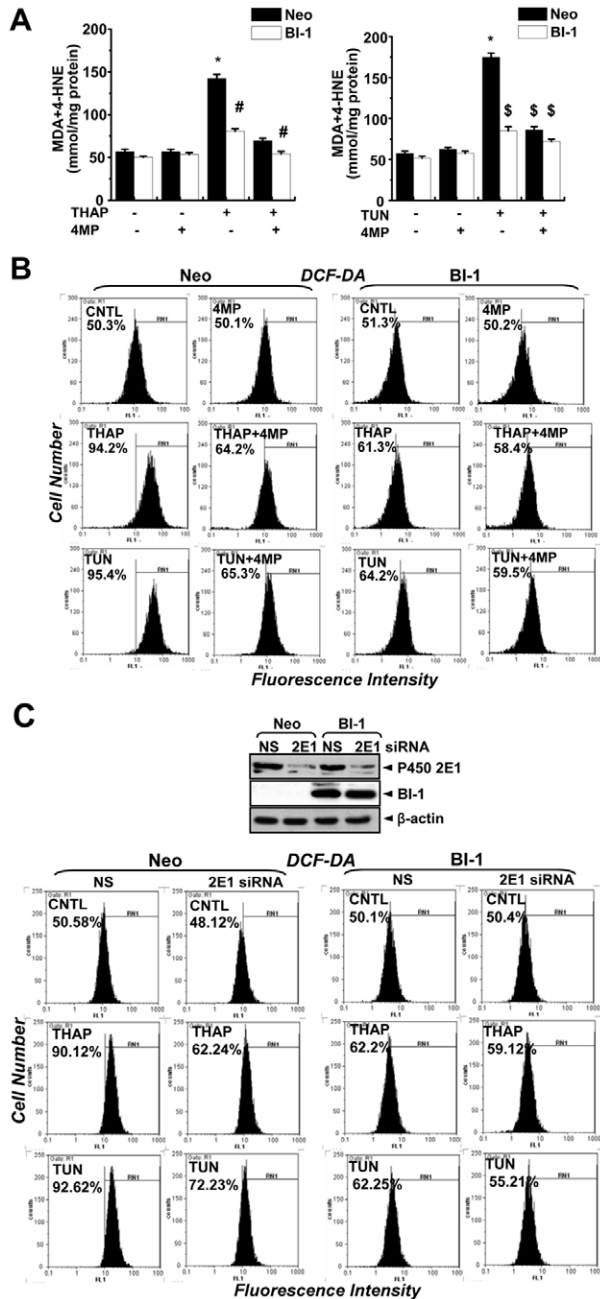


Fig. 3. Effect of a P450 2E1 inhibitor on ER stress-induced ROS accumulation. (A) Neo and BI1 cells were treated with either 5 μ M thapsigargin or 5 μ g/ml tunicamycin in the presence or absence of 1 mM 4-MP for 20 hours. The microsomal fractions were then isolated from the Neo and BI1 cells, and the ER lipid peroxidation was measured. * P <0.05, significantly different from the amount of malondialdehyde in the Neo cells; # P <0.05, significantly different from the amount of malondialdehyde in thapsigargin-treated Neo cells (left); # P <0.05, significantly different from the amount of malondialdehyde in tunicamycin-treated Neo cells (right). (B) Neo and BI1 cells were treated with either 5 μ M thapsigargin or 5 μ g/ml tunicamycin in the presence or absence of 1 mM 4-MP for 20 hours. DCFDA was loaded into the cells, and the fluorescence was measured. (C) Neo and BI1 cells were transfected with non-specific (NS) and P450 2E1 siRNA. Sixteen hours later, the expression of P450 2E1, BI1 and β -actin was analyzed by western blot (top). NS or P450 2E1 siRNA-transfected Neo and BI1 cells were treated with either 5 μ M thapsigargin or 5 μ g/ml tunicamycin for 20 hours. DCFDA was loaded into the cells, and the fluorescence was measured (bottom). MDA, malondialdehyde; CNTL, control; THAP, thapsigargin; TUN, tunicamycin; 4MP, 4-methylpyrazole.

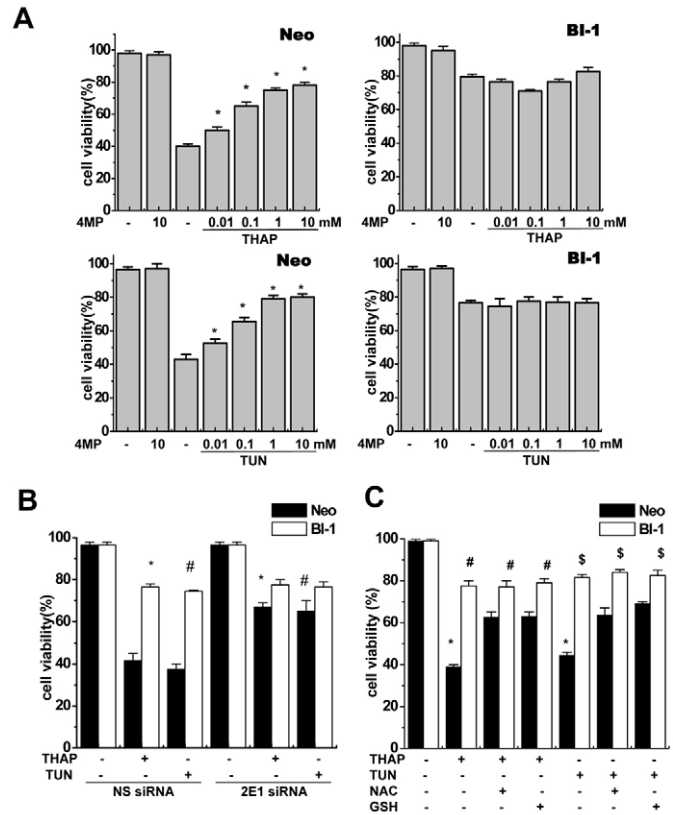


Fig. 4. Effect of P450 2E1 inhibitor on ER stress-induced cell death. (A) Neo and BI1 cells were treated with either 5 μ M thapsigargin or 5 μ g/ml tunicamycin in the presence or absence of the indicated concentrations of 4-MP for 48 hours, and cell death was measured as described in the Materials and Methods. The data shown indicate the mean \pm s.e.m. (n =6). (B) Non-specific or P450 2E1 siRNA-transfected Neo and BI1 cells were treated with either 5 μ M thapsigargin or 5 μ g/ml tunicamycin for 48 hours, and cell death was measured as described in the Materials and Methods. The data shown indicate the mean \pm s.e.m. (n =9). * P <0.05, significantly different from the viability in thapsigargin-treated Neo cells (NSsiRNA-transfected); # P <0.05, in tunicamycin-treated Neo cells (NSsiRNA-transfected). THAP, thapsigargin; TUN, tunicamycin; 2E1 siRNA, P450 2E1 siRNA; NS (siRNA), non-specific siRNA. (C) Neo and BI1 cells were treated with either 5 μ M thapsigargin or 5 μ g/ml tunicamycin in the presence or absence of 1 mM NAC or GSH for 48 hours, and cell death was measured as described in the Materials and Methods. The data shown indicate the mean \pm s.e.m. (n =4).

the production of H_2O_2 by P450 2E1 using a reconstitution system in the absence of BI1. Reconstituted P450 2E1 released H_2O_2 and promoted NADPH oxidation in the presence or absence of substrate (supplementary material Table S1). In human microsomes enriched with P450 2E1, similar patterns of NADPH oxidation and H_2O_2 release were observed (see supplementary material Table S1).

By contrast, when recombinant BI1 was incorporated into the reconstitution system by simple mixing of the soluble protein with DLPC liposomes (containing P450 and NPR), the production of H_2O_2 by P450 2E1 decreased with increasing concentrations of BI1 in an exponential decay manner, and eventually reached a state of equilibrium, in which only \sim 10% of H_2O_2 was produced at a protein ratio (BI1:NPR) of 1.5:2.0, when the amount of H_2O_2 in the sample without reconstituted BI1 was 100% (Fig. 5A).

To mimic conditions similar to those in vivo, the experiment was repeated with human microsomes enriched with P450 2E1. Adding

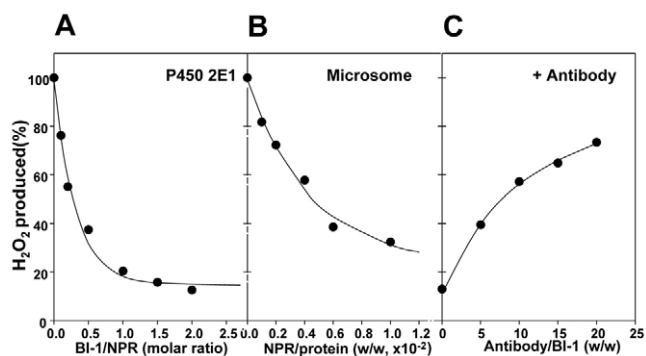


Fig. 5. Effect of BI1 on the production of H_2O_2 by P450 2E1. The amount of H_2O_2 produced was measured spectrophotometrically, with increasing ratios of BI1/NPR (A) or with increasing ratios of BI1/total amount of proteins in microsomes (w/w) (B). (C) Immuno-inhibition of BI1 induced H_2O_2 production was also measured with increasing ratios of antibodies (against the C-terminal region of BI1) to BI1 (w/w) at a BI1/NPR molar ratio of 2.0. During all experiments, the concentration of NPR was fixed at 0.8 μM .

increasing amounts of recombinant BI1 to the microsomes decreased the H_2O_2 levels (Fig. 5B). Although the presence of endogenous BI1 protein and the correct incorporation of recombinant BI1 into microsomes are unclear, this result indirectly implies that BI1 may act as an antioxidant in cells. To confirm the role of the C-terminal region of BI1 on the regulation of ROS production, we inhibited BI1 function using an antibody raised against the C terminus (AMNEKDKKKKEKK). The amount of H_2O_2 produced increased with increasing ratios of antibody to BI1 protein (Fig. 5C), but was not altered in the presence of anti-mouse serum (results not shown). This result suggests that the C-terminal region of BI1 is important in decreasing H_2O_2 production.

We then measured the production of H_2O_2 using an assay kit containing Amplex Red reagent, a fluorescent probe that is specifically oxidized by H_2O_2 in the presence of peroxidase. BI1 dose-dependently and linearly decreased the emission fluorescence of the probe when the reaction sample included P450 2E1 (Fig. 6A). As mentioned previously, P450 2E1 generated the greatest amount of H_2O_2 among the P450s. Together with the spectrophotometric data, these results indicate that BI1 reduces the production of H_2O_2 with increasing ratios of BI1/NPR. However, paralleling the result from the C-terminal neutralizing antibody treatment (Fig. 5C), recombinant deletion mutants of BI1 ($\Delta 8$ and $\Delta 16$) (supplementary material Fig. S3C) showed decreased inhibition of H_2O_2 production. In particular, in the $\Delta 16$ mutant, there was almost no effect on inhibition, emphasizing the importance of the C-terminal region of BI1 in the regulation of ROS production (Fig. 6B).

The experiment above was also repeated with another fluorogenic probe, Ac-Tempo, which specifically reacts with hydroxyl radicals and superoxide to emit fluorescence (Jiang et al., 2004; Touyz et al., 2003). Fluorescence occurred in the reconstitution system containing P450 2E1 in the absence of BI1 protein (supplementary material Fig. S4A, line a), indicating the release of radicals and superoxide but not absolute levels of ROS. Increasing the ratio of BI1/NPR decreased the fluorescence intensity of Ac-Tempo, suggesting that BI1 reduced the release of ROS (supplementary material Fig. S4A). ROS inhibition was lower in recombinant deletion mutants of BI1 (supplementary material Fig. S4B); however, the BI1 mutants did not show dramatic differences in membrane binding, suggesting that the diminished effect of ROS

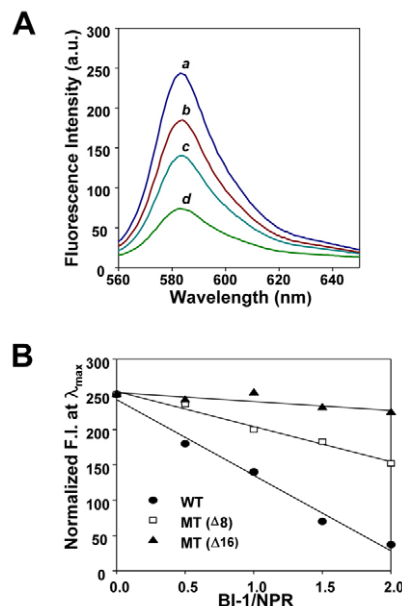


Fig. 6. Emission spectra of Amplex Red reagent. The amount of H_2O_2 generated from a reconstitution system containing P450 2E1 was measured using an Amplex Red assay kit according to the instructions of the manufacturer. (A) The emission spectra were recorded in the wavelength range of 560–650 nm, under an excitation wavelength of 545 nm. Lines a, b, c and d represent each of the emission spectra, which had BI1/NPR ratios of 0, 0.5, 1.0 and 1.5, respectively. (B) The λ_{max} (fluorescence intensity at 590 nm) was plotted with increasing BI1 (or BI1 deletion mutants)/NPR ratios. WT, MT ($\Delta 8$) and MT ($\Delta 16$) represent native BI1 and deletion mutants missing 8 and 16 amino acids at the C terminus, respectively.

inhibition in the BI1 mutants was not the cause. We next measured the effects of BI1 on the activities of catalase and horseradish peroxidase, without phospholipids or in a membrane-bound state. However, BI1 did not change catalytic activity in the presence of H_2O_2 (results not shown), suggesting that BI1 acts as a functional modulator of P450 2E1-induced oxidative stress.

BI1 interacts with NPR and P450 2E1

BI1 may directly interact with the NPR and/or P450 enzyme system. Therefore, we first tested the interaction of BI1 with NPR in a cell system. Co-transfection of BI1 (or Δ BI1: C-terminal deleted BI1) and NPR in 293T cells allowed co-immunoprecipitation of full-length BI1 and NPR, but not of Δ BI1 and NPR (Fig. 7A). This result suggests that BI1 and NPR bind each other *in vivo*, and that the C terminus of BI1 is required for binding. By contrast, direct binding between BI1 and P450 2E1 in the same system was relatively low (Fig. 7B). In addition, we examined the association of BI1 with NPR or P450 2E1 in a reconstitution system using fluorescence resonance energy transfer (FRET) analysis between Trp residues (NPR or P450 2E1) and IAEDANS (BI1). The fluorescence intensity at 490 nm increased with increasing NPR/BI1 ratios (Fig. 7C). Although the fluorescence intensity from increasing P450 2E1/BI1 ratios also increased (Fig. 7D), the intensity was much lower than that from NPR/BI1 (Fig. 7C). These results indicate that BI1 more efficiently interacts with NPR in a purified state. However, it is unclear how IAEDANS, a bulky fluorescent probe, influences the conformation of BI1 and thereby the physical contact between BI1 and NPR (or P450 2E1). As a control, the same FRET experiment was repeated in a soluble state without liposomes.

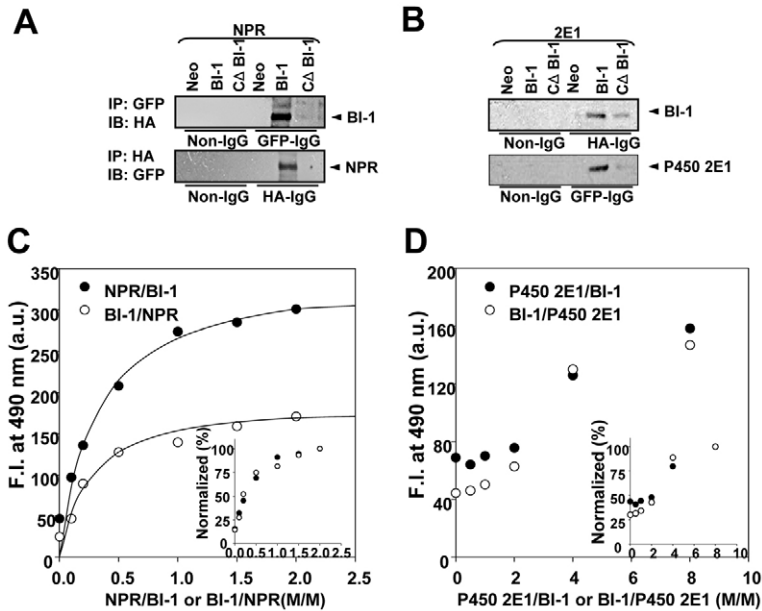


Fig. 7. The binding affinity between BII and NPR. (A) HEK293T cells were co-transfected with HA-BII and GFP-NPR or with HA-CA-BII (C-terminal deleted mutant) and GFP-NPR. (B) Cells were independently co-transfected with HA-BII and GFP-P450 2E1 or with HA-CA-BII and GFP-P450 2E1. Cell lysates were immunoprecipitated with anti-HA-antibody and immunoblotted with anti-GFP antibody. Using the reverse process, cell lysates were immunoprecipitated with anti-GFP antibody and immunoblotted with anti-HA antibody. In parallel, the cell lysates were immunoprecipitated with IgG. (C) The lysates were then immunoblotted with either anti-HA or anti-GFP antibody. IAEDANS-labeled BII and NPR were incorporated into liposomes, and the emission intensity at 490 nm was measured under an excitation wavelength of 290 nm to activate Trp fluorescence with increasing NPR/BII or BII/NPR ratios. (D) IAEDANS-labeled BII and P450 2E1 FRET assay was performed as for the assay of BII and NPR. In the normalized figure (inset), the fluorescence intensity at the protein ratio of 2.0 (in the case of FRET assay between BII and NPR) or 8.0 (between BII and P450 2E1) was set to 100%, and other data points were plotted relative to this point. Data shown indicate the mean \pm s.e.m. of five independent samples.

However, we were not able to measure any detectable energy transfer between BII and NPR, which suggests that the physical association of both proteins occurs only in a membrane-bound state.

In a physiological system, all of the NPR, P450 and BII reside on the ER membrane. NPR and P450 2E1 binding in the presence of BII is required for the MMO system to initiate oxygenation. The presence of BII altered the binding affinity between NPR and P450 2E1 (Fig. 8), suggesting the strong affinity between BII and NPR may induce dissociation between NPR and P450 2E1 through binding to either component. However, we could not exclude an interaction between BII and P450 2E1, although it would be weaker than between BII and NPR.

BII decreases the activity of P450 2E1

BII induced a change in the activity of P450 2E1, perhaps by altering the flow of electrons from NPR to P450 2E1. To test this possibility, we measured the activity of P450 2E1 as a function of NPR concentration. *p*-Nitrophenol and chlorzoxazone hydroxylase activities decreased with decreasing amounts of NPR (fixed levels of P450 2E1) (Fig. 9A). With *p*-nitrophenol, BII reduced P450 2E1 activity by ~60% at an NPR to P450 2E1 ratio of 0.5 (with catalytic activity in the absence of BII set at 100%).

In order to show more directly the effect of BII on P450 2E1 activity, we fixed the amounts of both NPR and P450 2E1, and only changed the amount of BII. Consistently, P450 2E1 activity was reduced as a function of BII concentration (Fig. 9B).

Discussion

The role of BII on P450 2E1 expression

BII inhibits ER stress-mediated ROS accumulation by regulating P450 2E1 activity. P450 2E1, a major source of ROS on the ER membrane, increased in response to the ER stresses, thapsigargin and tunicamycin (Fig. 2B). Our results are consistent with the theory that P450 2E1, which metabolizes and activates many toxicologically important substrates (including procarcinogens) into more toxic products, increases ROS and worsens pathology. To generalize the effect of BII on the modulation of ROS production, further work on other P450 subtypes, such as P450 1A2, another major source of

ROS formation, or other oxidative stress-generating systems should be performed. Another ER enzyme, HO-1, is increased after ER stress (Lee et al., 2007), but the increase occurs later than P450 2E1 (results not shown). Increased HO-1 expression is dependent on P450 2E1 (Gong et al., 2003) and perhaps acts as a compensatory survival signal against P450 2E1 expression. The relationship between P450 2E1 and HO-1 requires further study.

The mechanisms of BII-induced regulatory effects on ROS generation and cell death

BII may protect cells from the pathological effect of P450 2E1 by decreasing oxidative stress via two potential mechanisms. First, BII may enzymatically destroy ROS, as do catalase and horseradish peroxidase, although BII did not show this in our study. BII protein does not change molecular weight after ROS production, indirectly indicating that BII itself does not capture ROS and is not consequently oxidized.

The second possibility is that BII scavenges ROS produced by the P450 2E1 enzyme. The inhibition of P450 2E1 through chemical and siRNA approaches (Fig. 3) supports this possibility. Furthermore, the basal level of ROS seems to be lower in BII cells than in Neo cells, although the basal level was similar in Neo and BII cells (Fig. 3B,C; supplementary material Fig. S2B). The basal reductions in ROS in BII cells were consistent with lower expression of P450 2E1 in the BII cells (Fig. 1A), suggesting BII scavenges ROS produced by P450 2E1 even without stresses.

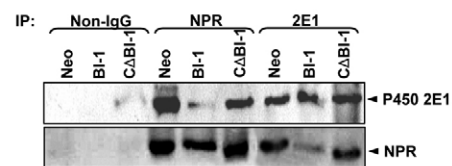


Fig. 8. The effect of BII on the association of NPR and P450 2E1. Neo, BII and CA-BII cells were immunoprecipitated with anti-NPR or anti-P450 2E1 antibodies or IgG. The lysates were then immunoblotted with either anti-NPR or anti-P450 2E1 antibodies.

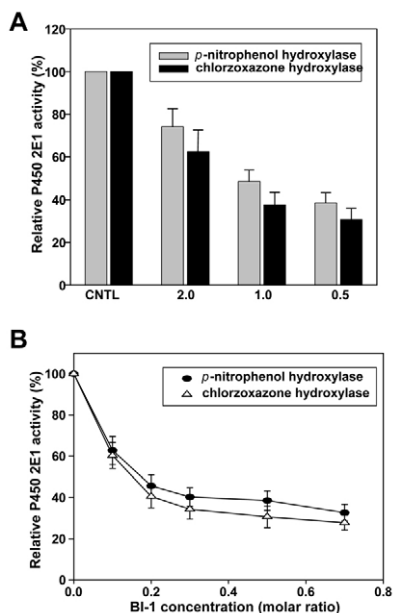


Fig. 9. Catalytic activities of P450 2E1. (A) The activities were measured with decreasing amounts of NPR incorporated into assay samples. Control represents the normalized catalytic activities of P450 2E1 at each indicated ratio of NPR/P450 in the absence of B11. Indicated numbers represent the ratio of NPR/P450 in the presence of equal molar concentrations of B11 and NPR. (B) The enzyme assays were repeated with increasing amounts of B11 in the presence of fixed concentrations of P450 2E1 and NPR (the ratio of NPR/P450 2E1 was 0.5). The y-axes represent the relative ratios of B11 to P450 2E1. CNTL, control.

ROS production induced by the ectopic expression of Bax is insensitive to the co-expression of AtB11 (Kawai-Yamada et al., 2004). However, BAX also increases mitochondria-initiated ROS accumulation and cell death. As B11 is expressed on the ER membrane (Xu and Reed, 1998), B11 may only directly regulate ER-originated ROS production. We believe that ER stress-associated ROS production is initiated from the ER and extends into the mitochondria, leading to cell death. Thus, the ectopic expression of BAX can be different from our ER stress-initiated ROS system.

As it was first documented that ER-resident caspase 12 mediates ER-specific apoptosis (Nakagawa et al., 2000), ER-specific or mitochondria-interconnected pathways have been actively studied (Zhang et al., 2008). ER stress-induced ROS production may be initiated from the ER, pushing mitochondrial ROS production above the threshold, leading to cell death. The mitochondria also play an important role in amplifying apoptotic signaling from the ER, where cytochrome c release plays an essential role (Zhang et al., 2008).

B11 was originally characterized as a protective protein that inhibited ER stress-mediated cytochrome c release but was less protective effect against cell death (Chae et al., 2004). Although it is premature to determine the specific step of B11-induced protection with these data alone, B11 may regulate ER stress-initiated ROS generation by interacting with the ER ROS production system, NPR and P450 2E1, and blocking the ER/mitochondria ROS amplification and apoptosis signaling.

The functional role of the physical association between B11 and NPR

B11 exists in close proximity and shows a higher affinity for NPR in vivo (co-immunoprecipitation between B11 and NPR in cells) and in a reconstitution system (FRET), than P450 2E1 (Fig. 6). B11 may induce dissociation of NPR and P450 2E1 and disrupt electron transfer between the two proteins, as a physical association between NPR and CYP family proteins is required for CYP activity and ROS production on the ER membrane.

Decreased levels of NPR decreased P450 2E1 catalytic activity more in the presence of B11, suggesting that B11 inhibits electron flow better when levels of NPR are limiting. In fact, P450s are present in the membrane in large excess compared with reductase, a limiting component in microsomes, with molar ratios ranging from 10:1 to 25:1 depending on treatment with inducers (Strobel et al., 1970). Therefore, the interaction of B11 with NPR may correlate with a decrease in P450 2E1-induced ROS by regulating electron flow.

The role of the B11 C-terminus on P450 2E1-induced ROS generation and NPR association

C-terminal deleted B11 showed less inhibition of P450 2E1-induced ROS generation (Fig. 5B; supplementary material Fig. S4B) and no NPR binding (Fig. 6A), but was still bound to the membrane in our model membrane system. Therefore, the C-terminal region is required for B11 activity, especially regulation of ROS and cell death.

Conclusion

In conclusion, B11 decreases electron uncoupling between NPR and P450 family proteins (especially P450 2E1), reducing ROS production and cell death.

Materials and Methods

Materials

Thapsigargin and tunicamycin were bought from Calbiochem (San Diego, CA). DCF-DA (2',7'-dichlorofluorescein diacetate) was obtained from Molecular Probes (Eugene, OR). The antibodies against P450 2E1, β -actin and the His₆ tag, as well as the siRNAs of NADPH-dependent p450 reductase and P450 2E1, were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). The sense and antisense strands of B11 and non-specific siRNA duplex are as follows: for B11, 5'-GUGGAAGGCCUUCU-UUCUA-3' (sense) and 5'-UAGAAAGAAGGCCUCCAC-3' (antisense); for non-specific control, CUGAACAAACCAUGCAAU-3' (sense) and 5'-AUUUGCAU-UGGUUGUUCAG-3' (antisense). The siRNAs were synthesized in duplex and purified forms using Bioneer technology (Daejeon, Korea). The Amplex Red assay kit was obtained from Invitrogen (Carlsbad, CA). Chlorzoxazone and p-nitrophenol were purchased from Sigma-Aldrich Co. (St Louis, MO). All phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). Dulbecco's modified Eagle's medium (DMEM) and other tissue culture reagents were supplied by Life Technologies (Grand Island, NY).

Generation of Neo and B11 stable cell lines

HA-pcDNA3-B11 and Neo-pcDNA3 plasmids (Neo: containing neomycin-selection marker) were transfected into 80% confluent HepG2 cells with lipofectamine (Invitrogen, CA). G418 (1 mg/ml; Wako) was used for selection. Neo and B11 stably transfected HEPG2 cell lines were generated and used during this study.

Expression and purification of recombinant proteins (P450 2E1, NPR and B11) and catalytic activity of P450 2E1

Recombinant human P450 2E1 was prepared as described previously (Winters and Cederbaum, 1992). Recombinant rat NPR was expressed in *E. coli* and purified as described previously (Shen et al., 1989; Hanna et al., 1998). Recombinant human B11 protein and its C-terminal deletion mutants ($\Delta 8$ and $\Delta 16$) containing an N-terminal (His)₆ tag were subcloned into the pRSETc expression vector (Invitrogen, CA). The B11 proteins were expressed and purified as described previously (Kim et al., 2008).

The activity of P450 2E1 was assayed by p-nitrophenol hydroxylation as described previously (Ahn et al., 2006) with slight modification. The standard incubation mixture (final volume of 0.5 ml) contained P450 2E1 (0.2 μ M), NPR (0.4 μ M), p-nitrophenol (100 μ M) and lipid vesicles (80 μ M) in 100 mM potassium phosphate buffer (pH 7.5). The catalytic activity of P450 2E1 was also determined by monitoring the hydroxylation of chlorzoxazone (Gillam et al., 1994).

DCF-DA assay

Intracellular ROS levels were measured as previously described (Kim et al., 2005). After treatment with ER stress agents, the cells were incubated with 100 μM 2',7'-dichlorofluorescein diacetate (DCF-DA) at 37°C for 30 minutes. The fluorescence intensity of 2',7'-dichlorofluorescein formed by a reaction between DCF-DA and intracellular ROS was analyzed by PAS flow cytometry (Partec, Münster, Germany) at excitation and emission wavelengths of 488 and 525 nm, respectively. Data are expressed as representative histograms from three independent experiments.

Microsomal fractionation

The microsomal fraction was obtained as previously described (Yang et al., 2004). Briefly, the cells were re-suspended in buffer A [250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA and 1 \times protease inhibitor complex (Roche Diagnostics, Mannheim, Germany)] on ice for 30 minutes. The cells were homogenized and the lysates centrifuged at 750 g for 10 minutes at 4°C to remove the non-lysed cells and nuclei. The supernatant was then centrifuged at 100,000 g for 1 hour at 4°C. The resulting supernatant was discarded, and the pellet was saved as the light membrane (LM:ER/microsome) fraction.

Western blotting and immunoprecipitation

Immunoblotting was performed as previously described (Kim et al., 2005). Immunoprecipitation was performed as previously described (Xu and Reed, 1998).

Labeling of proteins

Labeling of B11 or NPR with 1,5-IAEDANS was performed as previously described (Jeganathan et al., 2006). The amount of bound 1,5-IAEDANS was determined by absorption at 336 nm ($\epsilon_{336}=6100 \text{ M}^{-1} \text{ cm}^{-1}$) (Hudson and Weber, 1973).

Fluorescence resonance energy transfer (FRET)

All fluorescence measurements were performed at 30°C using a Shimadzu RF-5301 PC spectrofluorometer with a temperature-controlled cuvette. The energy transfer between Trp residues in NPR (or P450 2E1) and IAEDANS, a cysteine-specific fluorogenic probe, was measured in the emission range of 300–600 nm, with an excitation wavelength of 290 nm. Next, 0.4 μM NPR (or P450 2E1) was reconstituted into 100 μM of dilauroylphosphatidylcholine (DLPC) liposomes, including the IAEDANS-labeled B11 protein, whereas the ratio of NPR to B11 (or P450 2E1/B11, as a molar ratio) or B11 to NPR (or B11/P450 2E1) was increased.

Measurement of radicals and H₂O₂ formation

Reaction mixtures consisted of 0.4 μM P450 2E1, 0.8 μM NPR and 200 μM DLPC in the presence or absence of each substrate (100 μM) in 100 mM potassium phosphate buffer (pH 7.5). Reactions were initiated at 37°C by the addition of an NADPH-generating system (0.5 mM NADP⁺, 10 mM glucose 6-phosphate, and 1.0 IU glucose 6-phosphate dehydrogenase mL^{-1}) as described previously (Guengerich, 1994). The production of hydrogen peroxide (H₂O₂) was determined spectrophotometrically by reaction with ferroammonium sulfate and KSCN as described (Atkins and Sligar, 1988). The level of H₂O₂ was also measured fluorometrically using Amplex Red reagent (Hopper et al., 2006). The Amplex Red assay kit was used according to the manufacturer's instructions.

Polyclonal antibody preparation against B11 and immuno-inhibition

The epitope corresponding to the C terminus of B11 containing an additional Cys residue (CAMNEKDKKKEK) was used as an antigen, and antibodies against B11 were generated (Kim et al., 2008). Functional inhibition of B11 by these polyclonal antibodies was performed by preincubating B11 and the antibody with an increasing ratio of antibody to B11 without NPR in the reconstitution sample. After the addition of NPR, the amount of H₂O₂ produced was measured.

NADPH oxidation

NADPH oxidation was measured by reconstitution of P450 2E1 and NPR into DLPC, as described previously (Yun and Miller, 2000), in the presence or absence of B11.

Statistical analysis

Data from the dose-response experiments were analyzed by analysis of variance (ANOVA), as well as by two-tailed Student's *t*-tests. *P*<0.05 was considered significant. In each case, the statistical test used is indicated, and the number of experiments is stated individually in the legend of each figure.

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