The International Journal of Biochemistry & Cell Biology 44 (2012) 600–611

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Bax inhibitor-1 regulates the expression of P450 2E1 through enhanced lysosome activity

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ARTICLE INFO

Article history:
Received 29 October 2011
Received in revised form
20 December 2011
Accepted 22 December 2011
Available online 3 January 2012

Keywords:
BAX inhibitor-1
Lysosome
ROS
ER stress
P450 2E1

ABSTRACT

In this study, we explored the role of Bax inhibitor-1 (BI-1) on the expression of P450 2E1 and related ROS production. P450 2E1 protein, not mRNA, was expressed at relatively low levels in BI-1 plasmid-transfected cells (BI-1 cells) compared with neomycin-resistant vector-transfected cells (Neo cells). When exposed to ER stress, P450 2E1 expression and activity and ER membrane lipid peroxidation increased in both Neo cells and BI-1 cells, but to a lesser degree in BI-1 cells. This observation correlated with the lower level of ER stress in BI-1 cells than Neo cells. To examine the BI-1-associated P450 2E1 degradation mechanism, cells were treated with the lysosome inhibitor, bafilomycin and the proteasome inhibitor, MG132. Bafilomycin recovered the reduced P450 2E1 expression in BI-1 cells, but did not affect P450 2E1 expression in Neo cells. Next, proteosomal and lysosomal activities in Neo cells were compared to those in BI-1 cells. Although proteosomal activity was similar between Neo and BI-1 cells, LysoTracker and acridine orange labeling, lysosomal V-ATPase activity, and lysosomal cathepsin B expression were higher in BI-1 cells than in Neo cells. In the presence of ER stress, lysosomal activities decreased in Neo cells but did not change in BI-1 cells. P450 2E1 expression and ER membrane lipid peroxidation were greater in the hepatocytes and livers of BI-1 knock-out mice than in BI-1 wild-type cells and mice. Our results suggest that the BI-1-mediated enhancement of lysosomal activity regulates P450 2E1 expression and resultant ROS accumulation.

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1. Introduction

Bax inhibitor-1 (BI-1, also known as 'testis-enhanced gene transcript') is an anti-apoptotic protein that inhibits Bax activation and translocation to mitochondria (Xu and Reed, 1998). Functionally, BI-1 affects Ca^{2+} leakage from the endoplasmic reticulum (ER), as measured by Ca^{2+}-sensitive, ER-targeted fluorescent proteins and Ca^{2+}-sensitive dyes (Chae et al., 2004). Cells isolated from BI-$1^{-/-}$ mice exhibited hypersensitivity to apoptosis induced by ER stress (Chae et al., 2004), as well as the ER stress response after ischemia/reperfusion (Bailly-Maitre et al., 2006). It has been suggested that BI-1 may protect against ER stress (Bailly-Maitre et al., 2006; Lee et al., 2007; Krajewska et al., 2011). Studies of the protective mechanisms of BI-1 have focused on the regulation of reactive oxygen species (ROS), because ER stress-associated ROS accumulation is thought to be a mechanism of cell death (Kim et al., 2009; Park et al., 2010).

It was previously reported that BI-1 regulates the production of ROS by inhibiting Bax (Kawai-Yamada et al., 2004; Baek et al., 2004). In addition, it was previously shown that BI-1 overexpression increases heme oxygenase-1 (HO-1) expression, which may regulate ROS and ROS-associated cell death in response to ER stress (Lee et al., 2007). Even in the absence of ER stress, basally produced ROS levels are lower in BI-1-overexpressing cells (BI-1 cells) than in control cells, suggesting that elevated expression and activity of HO-1 in BI-1 cells may have a regulatory effect on endogenous ROS production. In addition, it has been suggested that BI-1 decreases electron uncoupling between NPR and P450 family proteins (especially P450 2E1), resulting in a reduction in ROS production (Kim et al., 2009). These previous findings highlight the

Abbreviations: BI-1, BAX inhibitor-1; ROS, reactive oxygen species; ER, endoplasmic reticulum; UPR, unfolded protein response; GRP78, glucose response protein 78; CHOP, C/EBP homologous protein; eIF-2α, eukaryotic initiation factor; JNK, c-Jun N-terminal kinase.

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doi:10.1016/j.biocel.2011.12.017

importance of determining the roles of P450 2E1, NPR, and HO-1 in BI-1-associated ROS regulation in more depth.

Cytochrome P450s constitute a large group of heme proteins that catalyze the oxidation of endogenous substrates such as steroids, and exogenous compounds such as drugs, toxicants, and procarcinogens. P450 2E1 is an example of a pro-oxidant cytochrome P450 (Cho et al., 2008; Cederbaum et al., 2009). Ethanol-inducible P450 2E1 is the most rapidly degraded of the P450s, with a short half-life of 6–7 h in the absence of substrate. Several studies have shown that loss of P450 2E1 is associated with ubiquitylation of the enzyme (Bush et al., 1997; Faouzi et al., 2007), although ubiquitylation was not observed in other reports (Correia et al., 2005; Han et al., 2005). A recent study reported the involvement of lysosomal and proteasomal activity in P450 2E1-mediated degradation (Wang et al., 2011). Recently, highly enhanced lysosomal activity was observed in BI-1-overexpressing adenocarcinoma cells (Lee et al., 2011a). The lysosomal activity of hepatocytes overexpressing BI-1, which also express P450 2E1, would therefore be of interest to determine.

We therefore explored how BI-1 regulates the expression of P450 2E1 and related ROS accumulation. Our results suggest that enhanced lysosome activity and associated P450 2E1 degradation in BI-1-overexpressing hepatic cells is one of the potential mechanisms of ROS regulation in this cell type.

2. Experimental procedures

2.1. Materials

The antibody against hemagglutinin antigen (HA) was purchased from Cell Signaling Technologies (Beverly, MA). Antibodies against the 20S core proteasome subunit and carbobenzoxy-Leu-Leu-Glu-7-amino-4-methyl coumarin (Z-LLE-AMC) were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and other tissue culture reagents were supplied by Life Technologies Inc. (Grand Island, NY, USA). Bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Biotechnology (Rockford, IL, USA). All other chemicals were of analytical grade or higher and were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Cell culture

HepG2 cells were stably transfected with pcDNA3 or pcDNA3-BI-1-HA plasmids using the Superfect transfection reagent (Qiagen, Hilden, Germany). The cells were then cultured for 3 weeks in 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA). Transfected human HT1080 fibrosarcoma cells were cultured in DMEM supplemented with 10% FBS, 20 mM HEPES, 100 μg/ml streptomycin, and 100 units/ml penicillin.

2.3. Animal experiments

The Animal Care Committee of Chonbuk National University Laboratory Animal Center approved our study protocol, and all experiments conformed strictly to committee guidelines. The handling of animals, including administration of drugs, tissue sampling, and euthanasia, was monitored by qualified animal care personnel.

2.4. Immunoblotting

Cell lysates were prepared, and the protein content of these lysates was measured as described in Kim et al. (2008). Equal amounts of protein extracted from cells with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS, and protease inhibitor cocktail) were separated on 10% SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes. After each membrane was probed with specific primary antibodies, the blot was stripped and re-probed with a polyclonal antibody against β-actin to confirm equal protein loading and transfer. An enhanced chemiluminescence system (ECL; Amersham-Pharmacia, Buckinghamshire, UK) was used for protein detection.

2.5. Lysosomal isolation

Lysosomal isolation was performed according to the protocol described in Lee et al. (2011b). Cells were rinsed in cold STE buffer (0.25 M sucrose, 0.01 M Tris–HCl, 1 mM EDTA, and 0.1% ethanol) and scraped into a dish containing 1 ml of STE buffer and protease inhibitors (Sigma–Aldrich). The cell suspension was placed in a Kontes cell disruption chamber and disrupted with three 20 min passes, each at 150 p.s.i. This method consistently disrupted >95% of cells, but left the lysosomes intact. The suspension was centrifuged at $1000 \times g$ to separate the post-nuclear supernatant from the nuclear pellet. The post-nuclear supernatant density was increased to 1.15 g/ml through the addition of sucrose and then applied to a sucrose density gradient ranging from 1.28 to 1.00 μg/ml. The gradient was centrifuged at $64{,}000 \times g$ for 4 h at 4 °C to separate lysosomal fractions based on buoyant density. The purity of the lysosomal preparation was assessed further by Western blotting for markers of cellular organelles, such as LAMP1. The isolated lysosomes were re-suspended in buffer containing 150 mM KCl to generate a high K^+ level inside the lysosome, which provided a membrane potential during stimulation of the V-ATPase with ATP.

2.6. ER membrane lipid peroxidation analysis

To measure ER membrane lipid peroxidation, the concentrations of the lipid peroxidation products, malondialdehyde (MDA) and 4-hydroxynonenal (4HNE), were measured using the BIOXYTECH LPO-586 commercial kit (Oxis International Inc., Portland, OR, USA) according to the manufacturer's protocol. The reactive aldehydic products of lipid peroxidation, MDA + 4-HNE, were measured in duplicate and expressed as nmol/mg of protein (Lee et al., 2011c). Separately, lipid hydroperoxide was measured using LPO assay (No. 705003) (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. The lipid hydroperoxide was measured in triplicate.

2.7. Real time analysis

Total RNA was extracted at the designated time points using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and 2 μg RNA was reverse transcribed using the Omniscript Reverse Transcription (QIAGEN, Valencia, CA, USA). Fluorescence-based real-time PCR was performed using the DNA Engine OPTICON® h2 system (MJ Research, Waltham, MA). SYBR green I Dye (Molecular Probes, Eugene, OR) and Go Taq® aFlexi DNA polymerase (Promega, Madison WI, USA) were used for PCR reactions. For quantification, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference for normalization of each sample. To determine P450 2E1 and NPR mRNA levels, real-time PCR was performed using the following primer pairs: P450 2E1 sense 5-TCAATCTCTGGACCCCAACTG3-, P450 2E1 antisense 5-GCGCTCTGCACTGTGCTTT-3, NPR sense: 5-ACCATTCCCACGTCTTCACATTTG-3, NPR antisense: 5-AGACATTCTCTCGTTCACCGCC-3.

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