Cytoprotective gene bi-1 is required for intrinsic protection from endoplasmic reticulum stress and ischemia-reperfusion injury

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Ischemia-reperfusion (IR) injury induces endoplasmic reticulum (ER) stress and cell death. Bax Inhibitor-1 (BI-1) is an evolutionarily conserved ER protein that suppresses cell death and that is abundantly expressed in both liver and kidney. We explored the role of BI-1 in protection from ER stress and IR injury by using bi-1 knockout mice, employing models of transient hepatic or renal artery occlusion. Compared to wild-type bi-1 mice, bi-1 knockout mice subjected to hepatic IR injury exhibited these characteristics: (i) increased histological injury; (ii) increased serum transaminases, indicative of more hepatocyte death; (iii) increased percentages of TUNEL-positive hepatocytes; (iv) greater elevations in caspase activity; and (v) more activation of ER stress proteins inositol-requiring enzyme 1 and activating transcription factor 6 and greater increases in expression of ER stress proteins C/EBP homologous protein and spliced XBP-1 protein. Moreover, hepatic IR injury induced elevations in bi-1 mRNA in wild-type liver, suggesting a need for bi-1 gene induction to limit tissue injury. Similar sensitization of kidney to ER stress and IR injury was observed in bi-1± mice. We conclude that bi-1 provides endogenous protection of liver and kidney from ER stress and IR injury. Analysis of components of the bi-1-dependent pathway for protection from IR injury may therefore reveal new strategies for organ preservation.

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ATF, activating transcription factor; BI-1, Bax Inhibitor-1; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; IR, ischemia reperfusion; IRE1, inositol-requiring enzyme 1; OGD, oxygen-glucose deprivation; sXBP, spliced XBP protein.

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Hepatocytes were cultured for 60 min in normal medium (C) or in medium containing 20 mM deoxyglucose (DG), 2.5, 5, or 10 mM KCN, or the combination (OGD), then washed and cultured 1 day in normal medium with or without 50 μM benzoyl-Val-Ala-Asp-fluoromethyl-ketone (z). As positive controls, hepatocytes were also treated with 10 μM staurosporine (STS) or 500 ng/ml anti-Fas (Fas) for 24 h. After treatments, the percentages of viable cells (A) (relative to control (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay) and TUNEL-positive cells (B) were quantified (mean ± SD; n = 5). (C and D) Hepatocytes were cultured for 1–3 h in medium lacking glucose in 1% O2, then washed and switched to normal medium and atmosphere, with or without 50 μM z. The percentages of viable (C) and TUNEL-positive (D) cells were quantified (mean ± SD; n = 4).

Hepatocytes by MTT assay (Fig. 1 A and C). Apoptosis was also monitored by TUNEL assay (Fig. 1 B and D).

Compared to wild-type hepatocytes, BI-1-deficient cells displayed significantly increased sensitivity to culture conditions that mimic IR injury, including KCN alone and KCN plus DG (Fig. 1 A and B). Also, bi-1 hepatocytes were more sensitive to hypoxia/glucose deprivation, with data for 2 h treatment reaching statistical significance (P < 0.05) (Fig. 1 C and D). Cell death induced by OGD was partially suppressed by benzoyl-Val-Ala-Asp-fluoromethyl-ketone (z), suggesting involvement of caspases. In contrast, BI-1 deficiency did not alter sensitivity to staurosporine (STS) or anti-Fas antibody (Fig. 1 A and B).

**BI-1 Deficiency Increases Hepatic IR Injury.** We tested the effects of BI-1 deficiency on sensitivity of liver to IR injury, employing a hepatic artery occlusion model (11). Using the Suzuki scoring method (0–4 scale) (12), histological analysis of livers from bi-1−/− and bi-1+/+ animals revealed more severe injury in BI-1-deficient animals (5.5 ± 0.5 vs. 3 ± 0.4; P < 0.05) (Supporting Text, Table 1, and Fig. 6, which are published as supporting information on the PNAS web site). In particular, the degree of necrosis was significantly higher in bi-1−/− mice than bi-1+/+ animals (P < 0.05). Histological lesions were absent in sham-operated animals.

We also evaluated the serum levels of enzymes released from dying hepatocytes in animals subjected to IR. At 90 min after hepatic ischemia, followed by 6 h of reperfusion, serum levels of aspartate (AST) and alanine (ALT) aminotransferases, as well as lactate dehydrogenase (LDH), were significantly higher in bi-1−/− mice compared to bi-1+/+ animals (AST, 4,712 ± 756 vs. 1,396 ± 183 units/liter, P < 0.05; ALT, 3,996 ± 795 vs. 1,251 ± 223 units/liter, P < 0.05; LDH, 12,727 ± 791 vs. 5,078 ± 80 units/liter, P < 0.05) (Supporting Text and Fig. 6). Thus, bi-1−/− mice demonstrate increased sensitivity to liver IR injury as determined by enzyme markers.

Finally, we evaluated neutrophil accumulation in the post-IR livers of bi-1−/− and bi-1+/+ mice as another indicator of the extent of injury. As measured by myeloperoxidase activity, neutrophil deposition 6 h after reperfusion in ischemic liver lobes was higher in bi-1−/− compared to bi-1+/+ mice, increasing from 0.04 ± 0.001 unit/g in sham controls to 0.74 ± 0.07 unit/g in bi-1−/− mice and 1.88 ± 0.36 units/g in bi-1−/− mice (P < 0.05) (Supporting Text and Fig. 6).

**BI-1 Deficiency Sensitizes Hepatocytes to IR-Induced Apoptosis.** To evaluate the effect of BI-1 deficiency on apoptosis in the IR model, TUNEL assays were performed by using liver sections from mice taken 6 h after reperfusion, detecting in situ cells with fragmented DNA indicative of apoptotic demise. The ischemic liver lobes of bi-1−/− mice contained statistically more TUNEL-positive hepatocytes (60 ± 9%) compared to wild-type animals (40 ± 9%) (P < 0.05) (Fig. 2 A). In contrast, virtually no TUNEL-positive liver cells were observed in non-ischemic liver lobes or in sham-operated animals (data not shown).

Because effector caspases are required for the execution phases of apoptosis, we investigated the activity of these proteases in ischemia-damaged liver 6 h after reperfusion. Asp-Glu-Val-Asp (DEVD)-hydrolyase activity in tissue lysates provides a measure of effector proteases such as caspases-3 and -7 (13). The rate of Asp-Glu-Val-Asp-amino-4-trifluoromethyl coumarin cleavage was much higher in homogenates of liver lobes (normalized for total protein content) derived from bi-1−/− mice compared to bi-1+/+ after IR injury (Fig. 2B).

Caspase-3 is typically activated by proteolytic processing mediated by upstream apoptotic proteases such as caspase-8 and caspase-9. Caspase-8 represents the apical caspase in the death receptor pathway (14), whereas caspase-9 serves as the initiator.
sequences of ER stress (18). When assessed by immunoblot lacking CHOP activity compromises cell viability, and cells factor whose expression is induced during ER stress and that comparable increases in Grp78 protein levels in both stream of these events, we monitored expression of these proteins whether the protection afforded by BI-1 maps upstream or down-
molecular chaperones, such as protein disulfide isomerase, Grp78, 2 statistical significance (Fig. 2

ER stress triggers activation of ER-resident protease, caspase-
12, in some scenarios (4). We therefore evaluated the levels and proteolytic processing of caspase-12 by immunoblotting but observed no changes (Fig. 3A), although many hepatocytes underwent apoptosis as measured by TUNEL assay (see above). Thus, it appears unlikely that caspase-12 plays a major role in the apoptotic program activated by IR injury in liver. The C/EBP homologous protein (CHOP) is a transcription factor whose expression is induced during ER stress and that participates in ER-mediated apoptosis (reviewed in ref. 17). Deregulated CHOP activity compromises cell viability, and cells lacking chop are significantly protected from the lethal consequ-
ences of ER stress (18). When assessed by immunoblot analysis in livers of control and BI-1-deficient mice, CHOP protein was elevated in untreated liver of bi-1/− mice compared to control bi-1/+ mice. After IR injury, CHOP protein became further elevated in bi-1/− mice but was barely detectable in bi-1/+ mice (Fig. 3B). Thus, BI-1-deficient mice produce more proapoptotic CHOP protein compared to normal animals before and after IR injury. c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase phosphorylate and enhance CHOP transcription and proapoptotic activity (17). We therefore examined the status of JNK and p38 in livers of bi-1/− and bi-1/+ mice before and after IR injury by using phospho-specific antibodies that serve as surrogate markers of kinase activity. Before IR injury, higher levels of phospho-JNK were detected in livers of bi-1/− mice compared to bi-1/+ animals (Fig. 3B). After IR injury, levels of phospho-JNK rose further in BI-1-deficient liver, whereas little phospho-JNK was detected in livers of wild-type mice. In contrast, no differences in phospho-p38 levels were observed in livers of either bi-1/− or bi-1/+ mice before or after IR injury. The bi-1/+ and bi-1/− mice also did not differ in their total levels of JNK and p38 proteins, as revealed by phospho-

Analysis of Hepatic Post-IR Events Associated with ER Stress and Apoptosis. It is known that IR injury promotes ER stress. During ER stress, increases occur in the expression of protective intra-ER molecular chaperones, such as protein disulfide isomerase, Grp78, and Grp94, intended to compensate for damage (4). To determine whether the protection afforded by BI-1 maps upstream or down-
stream of these events, we monitored expression of these proteins by immunoblotting in post-IR liver (Fig. 3A). IR injury induced comparable increases in Grp78 protein levels in both bi-1/− and bi-1/+ mice, indicative of ER stress. Protein disulfide bonding and Grp94 levels, however, were unchanged. ER stress triggers activation of ER-resident protease, caspase-
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Fig. 3. Analysis of molecular markers of ER stress. Mice were subjected to 90 min of hepatic ischemia, followed by 6 h of reperfusion. The ischemic (I) and normal liver lobes (NI) were then collected, and total proteins were extracted. The expression of ER stress and apoptotic proteins was compared in bi-1−/− and bi-1+/− livers by immunoblot analysis. Examples of representative blots are shown at the top. Quantification of the most relevant data were performed by scanning densitometry and is shown at the bottom (mean ± SEM; n = 3–7). Statistical significance was determined by t test and is denoted by asterisks (P ≤ 0.05).
Bi-1 Deficiency Increases Kidney Sensitivity to IR Injury. We previously demonstrated that bi-1−/− mice display increased sensitivity in vivo to tunicamycin-induced renal tubular toxicity, consistent with heightened sensitivity to ER stress-induced cell death in this organ (6). To extend these results, we determined the effect of Bi-1 deficiency on sensitivity to IR in the kidney, employing a renal artery occlusion model. At 45 min after renal ischemia, followed by 24 h of reperfusion, serum levels of creatinine and blood urea nitrogen (BUN) were significantly higher in bi-1−/− compared to bi-1+/+ animals (creatinine: 1.95 ± 0.26 vs. 0.43 ± 0.09 mg/dl, P < 0.005; BUN, 149.8 ± 10.4 vs. 48.3 ± 13.4 mg/dl, P < 0.0017). Thus, bi-1−/− mice demonstrate increased sensitivity to renal IR injury as determined by renal function markers. Using TUNEL assays to evaluate cell death in tissue sections, we observed greater sensitivity of bi-1−/− kidneys to IR injury, as revealed by a significant increase in the prevalence of TUNEL-positive cells in ischemic kidneys from bi-1−/− compared to bi-1+/+ mice (Fig. 4; see also Fig. 10, which is published as supporting information on the PNAS web site). Evidence of elevated apoptosis in bi-1−/− kidneys subjected to IR injury was also obtained by immunohistochemical analysis by using epitope-specific antibodies that recognized the cleaved (active) forms of caspase-3 and the caspase-3 substrate DNA fragmentation factor-45 (DFF45) (Figs. 4 and 9).

Fig. 4. Analysis of apoptosis markers in kidney from bi-1−/− and bi-1+/+ mice. (Upper Left) TUNEL-positive nuclei were quantified in tissue sections from kidney of bi-1−/− (KO) and bi-1+/+ (WT) mice and expressed as a percentage of total nuclei counted (mean ± SD; n = 4). The histograms summarize the expression levels of active caspase-3 and cleaved DFF40 as determined by immunoscore (Upper Center and Upper Right) and immunohistochemical densitometry (lower).
quence of more tissue injury, given the increased release of liver enzymes, more extensive necrosis, and increased incidence of TUNEL-positive hepatocytes seen in bi-1\(^{-/-}\) mice within hours after IR injury. Also, the results obtained by using cultured bi-1\(^{-/-}\) hepatocytes showing increased sensitivity to OGD also argue for a cell autonomous effect of BI-1 deficiency.

The mechanism by which the BI-1 protein protects hepatocytes from IR injury remains to be clarified. IR injury induces ER stress. The induction of ER chaperones was not significantly different in bi-1\(^{-/-}\) versus bi-1\(^{+/+}\) mice, indicating that the increased sensitivity of bi-1\(^{-/-}\) mice cannot be explained by differences in this particular ER stress response. However, BI-1-deficient liver and kidney showed evidence of activation of ER stress pathways under basal conditions, with further rises in pathway activation after IR injury, as manifested by increases in the levels of CHOP, sXBP-1, ATF6(N), and phospho-JNK (Fig. 11, which is published as supporting information on the PNAS web site). The sXBP-1 protein is produced as a downstream consequence of activation of the ribonuclease activity of IRE1 during ER stress, coupled with ATF6-mediated increases in XBP-1 mRNA production (26). Therefore, the increases in sXBP-1 observed in liver and kidney of bi-1\(^{-/-}\) mice could be a reflection of both ATF6 and IRE1 activation. These results suggest that absence of BI-1 protein predisposes to activation of ATF6 and IRE1 pathways during liver and kidney IR injury. Activation of ATF6 and IRE1 is known to increase expression of sXBP-1, ATF6(N), and phospho-JNK production and activation in vivo. These data thus expand recent evidence that bi-1 is required for intrinsic resistance to stroke injury (9), showing a broad role for this cytoprotective gene in protection from IR injury. Moreover, we show here that BI-1 deficiency results in an ER stress phenotype, as manifested by induction of CHOP, sXBP-1, ATF6(N), and phospho-JNK production and activation of IRE1. Analysis of components of the BI-1-dependent pathway for protection from ER stress and IR injury may reveal new strategies for organ preservation.

Materials and Methods

Mice. Mice with targeted disruption of the bi-1 gene (9) were used at 8 to 10 weeks of age, representing bi-1\(^{-/-}\) and bi-1\(^{+/+}\) littermates on a C57BL/6 background.

IR Injury Models. A warm hepatic IR model was used as described in ref. 11 with minor modifications. Arterial and portal venous blood supply were interrupted to the median and left lobes of the liver for 90 min, which was before reperfusion. Sham controls underwent the same surgical procedure but without vascular occlusion. Animals were killed 6 h after reperfusion. The renal IR injury model involved occlusion of the right renal artery and vein for 45 min (28). Animals were killed 24 h after surgery.

Hepatocyte Cultures. Hepatocytes were isolated from mouse liver and cultured as described in ref. 21. Hepatocytes were seeded into 24-well plates and cultured in normal medium or in medium containing 20 mM deoxyglucose and either 2.5 or 10 mM KCN to achieve OGD, then washed and cultured for 1 day in normal medium before performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays (29). Alternatively, cells were cultured with staurosporine (10 \(\mu\)M), anti-Fas mAb (500
buffered formalin, embedded in paraffin, sectioned (4–

ng/ml), or benzoyl-Val-Ala-Asp-fluoromethyl-ketone (50 μM)
(Sigma Chemicals). For some experiments, hepatocytes were
cultured in 60-mm plates (21) in Hanks’ Balanced Salt Solution
(with NaHCO₃) in a hypoxia chamber (1% O₂) for 1–3 h, then
washed and switched to normal media for 1 day in regular
atmosphere of 95% air and 5% CO₂.

Biomarkers and Immunohistochemical Assays. Methods for mea-
surement of serum markers of liver and kidney injury and
function, and assays for immunohistochemical analysis of apo-
tosis markers in tissues are provided in Supporting Text.

Histological Evaluation. Tissue specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned (4-μm
thick), stained with hematoxylin/eosin, and then analyzed
blindly. The severity of hepatic IR injury was graded by using
Suzuki’s criteria (ref. 12; Table 1). In this classification, sinusoi-
dal congestion, hepatocyte necrosis, and ballooning degenera-
tion are graded from 0 to 4.

Immunoblotting and Caspase Activity Assays. Detailed methods for
immunoblot analysis and caspase activity assays are provided as
Supporting Text. Briefly, liver and kidney homogenates were
examined by SDS/PAGE and immunoblotting by using various primary an-
tibodies in conjunction with an enhanced chemiluminescence
detection procedure or diluted into a caspase reaction buffer
containing fluorogenic caspase substrate peptides, with subse-
quent measurement of protease activity by spectrofluorimetry.

TUNEL Assay. Reperfused tissue specimens were frozen in OCT
compound and sectioned at 5 μm for processing by the TUNEL
method by using a commercial kit, employing DAB peroxidase
substrate (Roche Molecular Biochemicals) and counterstained
with 0.5% (wt/vol) methyl green. Specimens were evaluated by
UV microscopy at high power magnification (×400) in a blinded
fashion. A total of 30 random fields were counted for each
TUNEL-stained tissue sample.

Statistics. The data are expressed as mean ± standard deviation
(SD) from a minimum of three determinations. Statistical
significance of differences between various samples was deter-
mined by Mann–Whitney U test and t test.

We thank C. L. Kress, M. Thomas, and the personnel of the animal
facility at the Burnham Institute for Medical Research for technical
assistance: J. Chipuk (La Jolla Institute for Allergy and Immunology) for the anti-PUMA antibody; and D. Ron for valuable discussions. This work
was supported by the Californian Breast Cancer Research Program,
Fondation pour La Recherche Medicale, Philippe Fondation, and
National Institutes of Health Grant AG15393.

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Figure S1

A

WT

KO

I/R (X40) I/R (X100)

B

Total Suzuki's Score

WT KO

p < 0.05

C

AST

p < 0.05

SHAM WT KO

D

ALT

p < 0.05

SHAM WT KO

E

LDH

p < 0.05

SHAM WT KO

F

MPO

p < 0.05

SHAM WT KO
**Figure S2**

![Western blot images showing protein levels of CHOP, P-JNK, JNK, and Grp78 over hours of hypoxia for +/+, +/−, and −/− genotypes.](image)

**Figure S3**

![Bar graphs showing mRNA levels of Bi-1, formaldehyde, iNOS, eNOS, STAT 3, and eNOS for +/+, +/−, and −/− genotypes.](image)
Figure 4S

Protein

A

TNFα (pg/mL)

SHAM  WT  KO

B

IL-6 (pg/mL)

SHAM  WT  KO

C

IFN γ (pg/mL)

SHAM  WT  KO

RNA

D

mRNA levels

TNFα

+/+  +/+  +/−  −/−

E

mRNA levels

IL-6

+/+  +/+  +/−  −/−

F

mRNA levels

Rel-B

+/+  +/+  +/−  −/−

G

mRNA levels

Rel-A

+/+  +/+  +/−  −/−

* p < 0.05
Figure 5S