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 arterial 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.

Results

BI-1-Deficient Hepatocytes Are More Sensitive to Cell Death Caused by Oxygen-Glucose Deprivation (OGD). To explore the effects of BI-1 deficiency on the sensitivity of hepatocytes to IR, we used in vitro models of OGD. The first experimental paradigm includes an initial short phase of OGD created by culturing cells in deoxygenated media (1% O2) for 1–3 h in media without glucose, followed by a restoration phase. The second model involves placing the cells in a hypoxia chamber (1% O2) for 1–3 h in media without glucose, followed by a restoration phase. Accordingly, hepatocytes were isolated from bi-1-/- mice and bi-1+/+ livers (10) and subjected to OGD, then placed into fresh medium for 1 day, before assessing viability of

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-fluoromethylketone (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) (Supporting Text and Fig. 6). Data represent Vmax (mean ± SD; n = 5–8). Statistical significance (+) was determined by the Mann–Whitney U test (P < 0.05, KO versus WT).

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 provided a measure of effector proteases such as caspase-3 and -7 (13). The rate of Asp-Glu-Val-Asp-amino-4-trifluoromethyl coumarin cleavage was much higher in homogenates of liver lobes from bi-1−/− mice compared to bi-1+/+ mice (P < 0.05) (Fig. 2A). Caspase-3 activity in the liver of bi-1−/− mice was higher compared to bi-1+/+ mice (P < 0.05) (Supporting Text and Fig. 6). In contrast, virtually no TUNEL-positive liver cells were observed in non-ischemic liver lobes or in sham-operated animals (data not shown).

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caspase of the mitochondrial pathway (15). The preferred tetrapeptide substrates of caspases-8 and -9 are IETD and LEHD, respectively (16). We therefore evaluated levels of IETD and LEHD hydrolyase activity in liver from bi-1−/− and bi-1+/+ mice after IR injury. IR induced marked increases in the levels of both caspase-8 and -9 activity, as measured in liver homogenates (Fig. 2 C and D). The levels of caspase-9 activity, however, were significantly higher in liver of bi-1−/− compared to bi-1+/+ mice (Fig. 2C). In contrast, caspase-8 activity was slightly higher in the IR-damaged livers of bi-1−/− mice, but the results did not reach statistical significance (Fig. 2D).

**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 downstream 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-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 chom are significantly protected from the lethal consequences 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−/− compared to control bi-1+/+ animals. 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-independent antibodies (Fig. 3B).

XBP-1, ATF4, and ATF6 are transcription factors known to induce chom during ER stress (17, 19). Activated IRE1 initiates unconventional splicing of the mRNA encoding an isoform of XBP-1 protein that induces expression of chom (17). Activated PKR-like ER kinase phosphorylates eIF2, on serine-51A, which results in translocational induction of ATF4 (20). ATF6 is cleaved during ER stress, and its cytosolic domain [ATF6(N)] translocates to the nucleus. We therefore examined the status of XBP-1, ATF4, and ATF6 in livers of bi-1−/− and bi-1+/+ mice before and after IR injury. Levels of spliced XBP-1 protein and cleaved ATF6 were higher in livers of bi-1−/− mice before and after IR injury (Fig. 3C). After IR injury, sXBP-1 protein levels in bi-1−/− mice approximately doubled but remained undetectable in bi-1+/+ animals. In contrast, differences in eIF2α phosphorylation were not detected in bi-1−/− and bi-1+/+ mice before or after IR injury by using phospho-specific antibodies (Fig. 3C). Consistent with this result, levels of ATF4 remained unchanged after IR in both bi-1−/− and bi-1+/+ mice (data not shown). These observations suggest that BI-1 deficiency enhances ATF6 processing and increases selected IRE1 activities (ribonuclease activity but not caspase-12 activation), whereas activation of PKR-like ER kinase pathway is not affected by loss of BI-1. Portions of these data were confirmed by using cultured hepatocytes subjected to hypoxia/glucose deprivation, revealing con-
stutively elevated levels of CHOP, P-JNK, and ATF6 (but not Grp78, Grp94, or ATF4) in bi-1 cells (Fig. 7, which is published as supporting information on the PNAS web site).

Members of Bcl-2 family play important roles in the regulation of apoptosis and cell death, and some of these proteins target not only mitochondrial but also ER membranes (reviewed in ref. 4). We therefore analyzed by immunoblotting the expression of selected Bcl-2-family proteins in liver before and after IR injury. Because the antiapoptotic protein Bcl-2 is not constitutively expressed in mouse liver (10), we studied other family members. Bcl-Xs is the major antiapoptotic protein expressed in hepatocytes (21) and, thus, its expression was evaluated. In addition, we evaluated expression of Bcl-Xr, a prosapoptotic product of the bcl-X gene that arises through alternative mRNA splicing (22) by using an antibody that preferentially reacts with this isoform of Bcl-X (23). Furthermore, because hypoxia, IR injury, or ER stress has been reported to induce expression of prosapoptotic Bcl-2-family proteins Nip3, Bax, and PUMA (24), we also examined these proteins by immunoblotting. Levels of antiapoptotic Bcl-XL and prosapoptotic Nip3, Bax, and PUMA were not detectably different in the livers of bi-1−/− and bi-1+/+ mice before or after IR injury. In contrast, IR injury induced increases in the prosapopotic Bcl-Xr protein in livers of both bi-1−/− and bi-1+/+ mice after IR injury, with the extent of induction significantly higher in BI-1-deficient animals compared to wild-type mice (Fig. 3D). Thus, although BI-1 deficiency has no effect on expression of most Bcl-2-family proteins examined, BI-1 deficiency may promote increased induction of Bcl-X, during liver IR injury.

IR Injury Induces bi-1 Gene Expression. To determine whether bi-1 gene expression is affected by IR injury, we evaluated intrapathic bi-1 mRNA levels in wild-type mice, contrasting nonischemic (NI) and ischemic (I) areas. After IR, livers of mice contained significantly increased hepatic bi-1 mRNA levels, as compared with nonsignificant controls (390 ± 77 vs. 200 ± 61 arbitrary units [AU]; P < 0.05) (Fig. 8, which is published as supporting information on the PNAS web site). In contrast, levels of STAT3 and RelA mRNA were not substantially different, serving as a specificity control. Thus, bi-1 may be a cytoprotective component of the cellular response to hypoxia adaptation, making it a candidate mediator of preconditioning.

Recent data suggest that endothelial nitric oxide synthase (eNOS) is important in limiting the extent of postischemic liver injury, whereas inducible NOS (iNOS) exacerbates it (25). We therefore compared expression of constitutive (eNOS) and inducible (iNOS) mRNA levels after hepatic IR injury in bi-1+/+ and bi-1−/− mice. Hepatic expression of iNOS was higher in bi-1−/− mice subjects to IR injury (1,090 ± 313 vs. 201 ± 50 a.u.; P < 0.05), whereas eNOS mRNA levels were comparable in bi-1+/+ and bi-1−/− animals (3,913 ± 1,077 vs. 3,040 ± 705 a.u.; P = nonsignificant) (Fig. 9, which is published as supporting information on the PNAS web site). We conclude, therefore, that the increased sensitivity of bi-1−/− mice to IR injury cannot be explained by deficient eNOS expression. In contrast, the higher iNOS expression in postischemic bi-1−/− livers correlates with greater tissue damage in these mice but may be a secondary consequence of the more extensive cytokine elaboration that occurs in BI-1-deficient mice (Fig. 9) as a result of more extensive necrosis and inflammatory cell infiltration (Fig. 6).

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).

Analysis of IR-Associated Changes in ER Stress Proteins in Kidney. Because elevated levels of CHOP, sXBP-1, ATF6(N), and phospho-JNK were observed in livers of bi-1−/− mice, we analyzed the levels of these proteins in the kidneys from bi-1−/− versus bi-1+/+ mice before and after IR. Before IR injury, levels of these proteins were similar in the kidneys of bi-1−/− and bi-1+/+ animals (Fig. 5). After IR, marked increases in CHOP, sXBP-1, ATF6(N), and phospho-JNK were found in kidneys of bi-1−/− but not bi-1+/+ mice. In contrast, differences in phospho-p38 and phospho-eIF2a were not observed in bi-1−/− and bi-1+/+ animals. We conclude therefore that BI-1 is required for suppression of ER stress pathways that lead to expression of CHOP and sXBP-1 proteins and that are responsible for activation of ATF6, IRE1, and JNK in both kidney and liver.

Discussion

The data reported here show that loss of bi-1 sensitizes both liver and kidney to IR injury, thus establishing an important in vivo role for this gene in intrinsic resistance to IR-induced cell loss and tissue damage in these organs. Although we favor a direct cytoprotective role for BI-1, we cannot exclude the possibility that secondary factors contribute to the increased sensitivity of liver and kidney to IR injury in vivo. For example, the post-IR livers of bi-1−/− mice accumulated more inflammatory cells and more intrahepatic production of TNFα and IL-6 mRNA was detected, along with higher levels of circulating TNFα and IL-6 in serum. Thus, it is conceivable that bi-1 is required for suppressing inflammatory responses in vivo, and absence of this gene therefore results in unrestrained inflammatory cell activity. However, it is more likely that the increased inflammatory response observed in bi-1−/− mice after IR injury is a conse-
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-I/−/− mice within hours after IR injury. Also, the results obtained by using cultured bi-I/−/− 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-I/−/− versus bi-I/+/+ mice, indicating that the increased sensitivity of bi-I/−/− 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-I/−/− 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 I/R injury. Activation of ATF6 and IRE1 is known to increase expression of chop (gadd153), a member of the C/EBP-family of basic leucine zipper transcription factors that induces apoptosis (reviewed in ref. 17). In addition to inducing sXBP-1 production, IRE1 activation also induces JNK activation as a downstream consequence of recruiting TRAF2 and activating Ask1 (27). Thus, the increases in phospho-JNK observed in liver and kidney of bi-I/−/− mice could be another reflection of IRE1 activation. Interestingly, JNK is speculated to promote CHOP activity at a posttranscriptional level by increasing its transcriptional activity through phosphorylation (17), thereby potentially reinforcing the sXBP-1-dependent pathway that increases CHOP production. How loss of BI-1 encourages activation of the IRE1 and ATF6 pathways remains to be determined. Among the possibilities are effects of BI-1: (i) on ER Ca2+-affecting Ca2+-regulated ER chaperones that suppresses IRE1 and ATF6, and (ii) direct interactions of BI-1 with IRE1 and ATF6 proteins complexes in ER membranes. These and other possible mechanisms require future experimental investigation.

Interestingly, we observed that IR injury induces bi-I expression in normal liver. We hypothesize, therefore, that induction of endogenous bi-I gene expression represents an important adaptive response that limits tissue injury during IR. This finding raises the possibility that bi-I could play a role in the phenomenon of preconditioning, where transient episodes of reversible ischemia provide protection from subsequent hypoxic events. Further experimentation is required to test this hypothesis.

In summary, we have demonstrated the bi-I gene is required for intrinsic resistance to IR injury in the mammalian liver and kidney in vivo. These data thus expand recent evidence that bi-I 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-I gene (9) were used at 8 to 10 weeks of age, representing bi-I/−/− and bi-I/+/+ 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 μM), anti-Fas mAb (500 μM), and anti-Fas mAb (500 μM).
buffered formalin, embedded in paraffin, sectioned (4-μm thick), stained with hematoxylin and 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, sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are graded from 0 to 4.

**Biomarkers and Immunohistochemical Assays.** Methods for measurement of serum markers of liver and kidney injury and function, and assays for immunohistochemical analysis of apoptosis 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. In this classification, sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration 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 normalized for total protein content and either subjected to SDS/PAGE and immunoblotting by using various primary antibodies in conjunction with an enhanced chemiluminescence detection procedure or diluted into a caspase reaction buffer containing fluorogenic caspase substrate peptides, with subsequent 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 determined 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.
Figure S2

Figure S3

**Bi-1**

- NI
- +/+ 
- +/- 

**p < 0.05**

**iNOS**

- NI
- +/+ 
- +/- 

**p < 0.05**

**mRNA levels**
Figure S2

Figure S3
Figure 4S

Protein

**A**

- **TNFα** (pg/mL)
- **p < 0.05**

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**B**

- **IL-6** (pg/mL)
- **p < 0.05**

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**C**

- **IFNγ** (pg/mL)

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RNA

**D**

- **TNFα**
- **p < 0.05**

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**E**

- **IL-6**
- **p < 0.05**

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**F**

- **Rel-B**
- **p < 0.05**

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**G**

- **Rel-A**

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Figure 5S
Translation Induction of ATF4

Perk

ATF6

Bi-1

IRE1

BI-1

Unspliced XBP1 mRNA

Spliced XBP1 mRNA

pXBP1(S)

CHOP

Apoptosis

ATF6(N)

ERSE

NF-Y

ERSE

AARE

CHOP

NF-κB

Tran

α

TRAF2

Casp12

JNK

ASK1

NF-κB

Casp12