Supplementary Data

Supplementary Materials and Methods

Protein carbonylation assay

Carbonylation of liver proteins (10 μg) was examined by an analysis of tissue homegenates with an Oxyblot Protein Oxidation Detection Kit or an OxyELISA Oxidized Protein Quantitation Kit (Chemicon).

Lipid peroxidation analysis

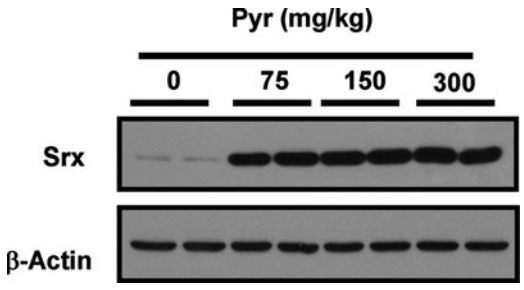
Liver tissue fixed with 10% buffered formalin was embedded in paraffin and sectioned. Sections were then depleted of paraffin and subjected to an immunohistochemical analysis with antibodies to 4-hydroxynonenal (JaICA) with the use of an ABC staining kit (Vector Laboratories). Liver homogenates (20 μg of protein) were assayed for malondialdehyde with the use of a thiobarbituric acid–reactive substance (TBARS) assay kit (Cayman Chemical).

Sulfiredoxin and peroxiredoxin III knockout mice

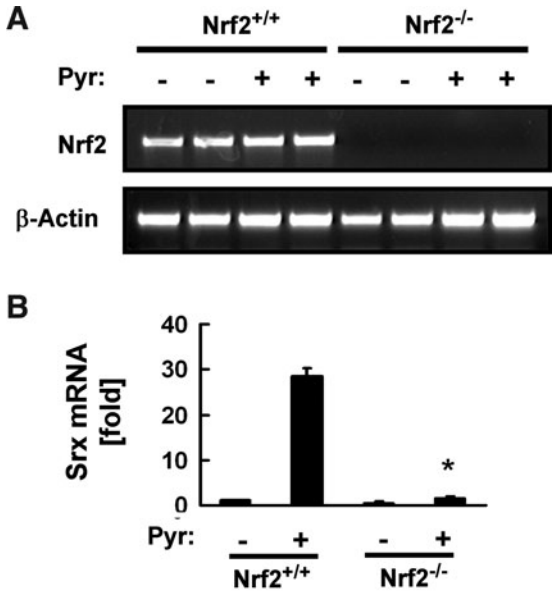
The sulfiredoxin $(Srx)^{flox/flox}$ was previously described (1). To generate systemic Srx knockout mice, we bred the $Srx^{flox/flox}$ mice with mice expressing a Cre recombinase transgene under the control of the PGK gene promoter (The Jackson Laboratory). The resulting $Srx^{flox/flox}$:PGK-Cre mice were designated $Srx^{-/-}$ mice. For the generation of peroxiredoxin (Prx) III knockout mice, a DNA fragment containing the PrxIII gene was obtained from 129/SvJ mouse genomic DNA by polymerase chain reaction. A targeting vector was designed to replace a ~5.1-kb genomic fragment containing exons 1 to 4 of the PrxIII gene with the PGK-neo selection cassette. The targeting vector was linearized and introduced by electroporation into 129/SvJ mouse J1 embryonic stem cells. Clones resistant to G418 and gancyclovir were selected, and two clones containing the introduced mutation were separately injected into C57BL/6 mouse blastocysts, which were then transferred to pseudopregnant foster mothers. The resulting male chimeric mice were bred with C57BL/6 females to obtain mice heterozygous for the mutant PrxIII allele, which were, in turn, crossed to generate homozygous PrxIII knockout mice.

Cell culture and measurement of calcium by Rhod-2

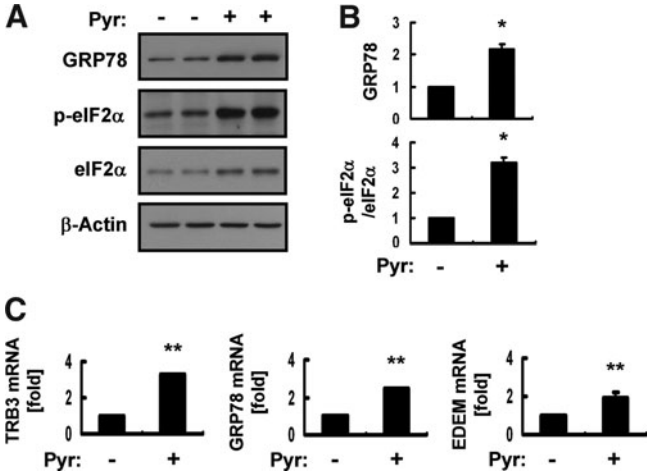
HeLa and Hepa1c1c7 cells were maintained in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. The culture medium consisting of HeLa and Hepa1c1c7 cells plated on cover slips was replaced with a physiological salt solution (PSS: 140 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* glucose, 10 m*M* HEPES-NaOH, pH 7.4) containing 10 μM of the acetoxymethyl ester of Rhod-2 (Invitrogen) and 10% Pluronic F127, and the cells were incubated for 30 min at room temperature. The cells were washed once with PSS, perfused on the stage of a microscope (Zeiss Axio Observer) with PSS at room temperature and a rate of 10 to 12 ml/min for at least 5 min, and then stimulated with 10 μM A23187, 25 μM pyrazole, dimethyl sulfoxide (solvent for A23187), or phosphate-buffered saline (solvent for pyrazole). The changes in Rhod-2 fluorescence were monitored. Changes in Rhod-2



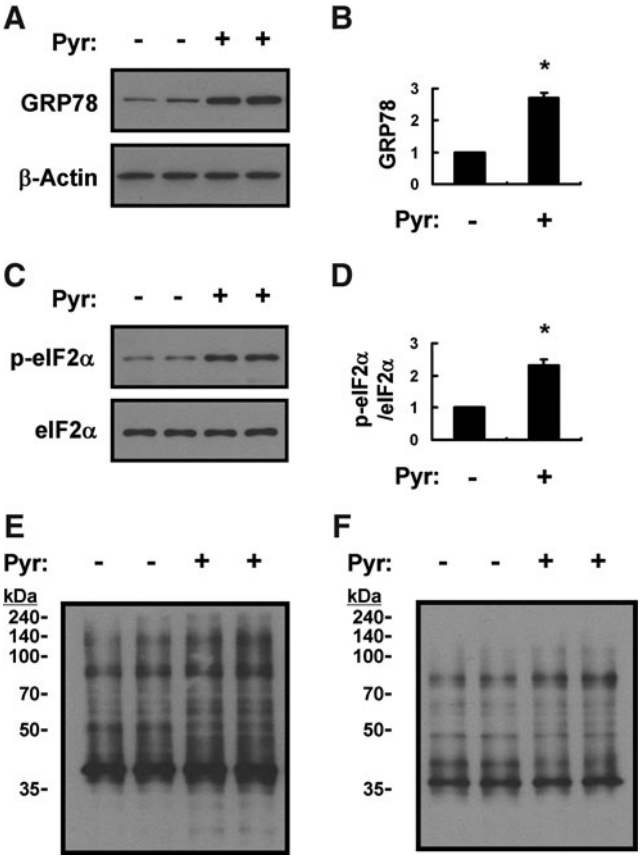
SUPPLEMENTARY FIG. S1. Concentration-dependent induction of Srx in the liver of pyrazole-treated mice. Liver homogenates (20 μg of protein) prepared from mice 18 h after an intraperitoneal injection of the indicated doses of Pyr were subjected to an immunoblot analysis with antibodies specific for Srx and for β-actin. Pyr, pyrazole; Srx, sulfiredoxin.



SUPPLEMENTARY FIG. S2. RT-PCR analysis of Nrf2 mRNA and RT-real-time PCR analysis of Srx mRNA in $Nrf2^{-/-}$ and wild-type mice. (A) Total RNA extracted from the liver of $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice 18 h after an intraperitoneal injection of saline (−) or pyrazole (150 mg/kg) was subjected to RT-PCR analysis with primers specific for Nrf2 or β-actin (internal control). Each lane corresponds to a different animal. **(B)** Total RNA prepared from the liver of mice treated as in **(A)** was subjected to RT-real-time PCR analysis for determination of the amount of Srx mRNA. Data are means ± SD for three mice and are expressed relative to the corresponding value for the saline-treated $Nrf2^{+/+}$ mice. *$p < 0.03$ versus pyrazole-treated $Nrf2^{+/+}$ mice. Nrf2, nuclear factor erythroid 2–related factor 2; RT-PCR, reverse transcription–polymerase chain reaction; SD, standard deviation.



SUPPLEMENTARY FIG. S3. Pyrazole induces ER stress in mouse liver. (A) Liver homogenates (20 μg of protein) prepared from mice 18 h after an intraperitoneal injection with saline (−) or pyrazole (150 mg/kg) were subjected to an immunoblot analysis with antibodies specific for GRP78, for phosphorylated (p-) or total forms of eIF2α, or for β-actin. Data are representative of three independent experiments. **(B)** Densitometric analysis of the abundance of GRP78 (normalized by that of β-actin) and that of phosphorylated eIF2α (normalized by that of total eIF2α) in immunoblots similar to those in **(A)**. Data are expressed relative to the corresponding value for saline-injected mice and are means ± SD. $^*p < 0.05$ versus the saline-treated group. **(C)** Total RNA prepared from the liver of mice treated as in **(A)** was subjected to RT and real-time PCR analysis for the determination of the amounts of TRB3, GRP78, and EDEM mRNAs. Data are means ± SD and are expressed relative to the corresponding value for the saline-treated group. $^{**}p < 0.03$ versus saline-treated mice. EDEM, ER degradation–enhancing α-mannosidase–like protein; eIF2, eukaryotic initiation factor 2; ER, endoplasmic reticulum; GRP, glucose-regulated protein.



SUPPLEMENTARY FIG. S4. Pyrazole induces ER stress in HeLa and Hepa1c1c7 cells. (A) HeLa cells were treated with pyrazole (100 μM), and cell homogenates were subjected to an immunoblot analysis with antibodies specific for either GRP78 or β-actin. Data are representative of three independent experiments. **(B)** Densitometric analysis of the abundance of GRP78 (normalized by that of β-actin) in immunoblots in **(A)**. Data are means ± SD for triplicates. $^*p < 0.05$ versus the control group. **(C)** Hepa1c1c7 cells were treated with pyrazole (100 μM), and cell homogenates were subjected to an immunoblot analysis with antibodies specific for phosphorylated (p-) or total forms of eIF2α. Data are representative of three independent experiments. **(D)** Densitometric analysis of the abundance of phosphorylated eIF2α (normalized by that of total eIF2α) in immunoblots in **(C)**. Data are means ± SD for triplicates. $^*p < 0.05$ versus control group. **(E)** Homogenates (20 μg of protein) from HeLa cells treated as in **(A)** were subjected to an immunoblot analysis of carbonylated proteins with the use of an Oxyblot Protein Oxidation Detection Kit (Chemicon). Molecular size markers are indicated in kilodaltons. Data are representative of three independent experiments. **(F)** Homogenates (20 μg of protein) from Hepa1c1c7 cells treated as in **(C)** were subjected to an immunoblot analysis of carbonylated proteins with the use of an Oxyblot Protein Oxidation Detection Kit (Chemicon). Molecular size markers are indicated in kilodaltons. Data are representative of three independent experiments.

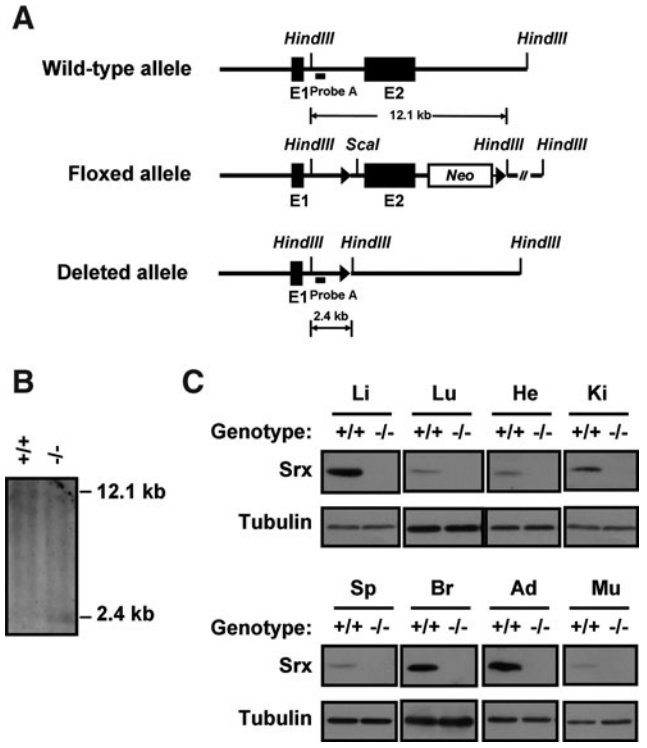
fluorescence over 30 min were monitored at an excitation and emission wavelengths of 540 and 595 nm, respectively. The emitted fluorescence was captured with a CCD camera (Roper Scientific) and analyzed with the use of Metafluor software (Universal Imaging Corp).

Detection of mitochondrial reactive oxygen species

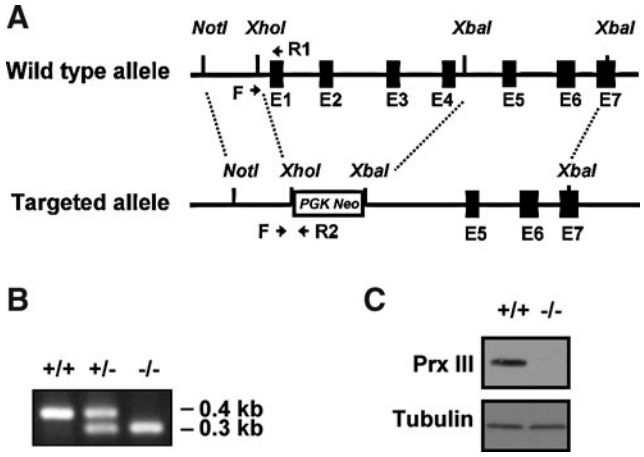
Mitochondrial-specific reactive oxygen species generation was measured using the MitoSox Red fluorescence dye (Molecular Probed) as described by the manufacturer. The fluorescence intensity was measured by FACSCalibur flow cytometry (BD Biosciences).

Supplementary Reference

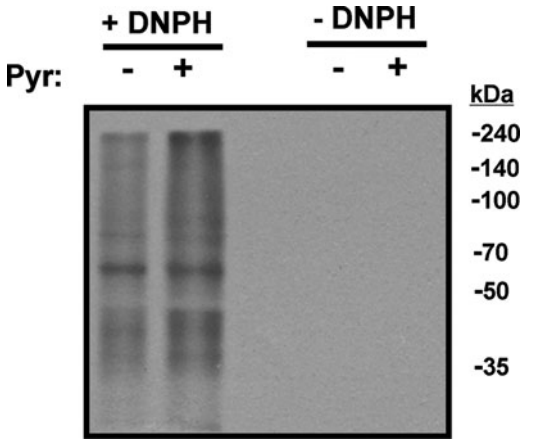
1. Bae SH, Sung SH, Cho EJ, Lee SK, Lee HE, Woo HA, Yu D, Kil IS, and Rhee SG. Concerted action of sulfiredoxin and peroxiredoxin I protects against alcohol-induced oxidative injury in mouse liver. *Hepatology* 53: 945–953, 2011.



SUPPLEMENTARY FIG. S5. Generation of conditional Srx knockout mice. (A) Schematic representations of the wild-type, floxed, and deleted alleles of the mouse Srx gene. **(B)** For confirmation of the Cre-mediated deletion of the floxed allele of the Srx gene, genomic DNA isolated from the tail of mice was digested with HindIII and subjected to Southern blot analysis with probe A. HindIII fragments corresponding to the wild-type (12.1 kb) and deleted (2.4 kb) alleles are indicated for mice of the indicated genotypes. **(C)** Homogenates (20 μg of protein) of the liver (Li), lung (Lu), heart (He), kidney (Ki), spleen (Sp), brain (Br), white adipose tissue (Ad), and skeletal muscle (Mu) prepared from $Srx^{+/+}$ or $Srx^{-/-}$ mice were subjected to an immunoblot analysis with antibodies specific for either Srx or Tubulin.



SUPPLEMENTARY FIG. S6. Generation of PrxIII knockout mice. (A) Schematic representations of the wild-type allele of the mouse PrxIII gene. *Arrows* indicate the PCR primers used for detection of the wild-type (F and R1) and mutant (F and R2) alleles. **(B)** Genomic DNA extracted from the tail of engineered mice was genotyped by PCR with the primers indicated in **(A)**. The sizes of PCR products specific for the wild-type and mutant alleles are 0.4 and 0.3 kb, respectively. **(C)** Liver homogenates (20 μg of protein) prepared from $PrxIII^{+/+}$ or $PrxIII^{-/-}$ mice were subjected to an immunoblot analysis with antibodies specific for PrxIII and Tubulin. Prxs, peroxiredoxins



SUPPLEMENTARY FIG. S7. Detection of carbonylated proteins in liver homogenates. Liver homogenates (20 μg of protein) prepared from mice 18 h after an intraperitoneal injection of saline (−) or pyrazole (150 mg/kg) were subjected to derivatization in the presence or absence of DNPH followed by an immunoblot analysis with antibodies specific for DNPH. DNPH, 2,4-dinitrophenylhydrazine.