Scavenger Receptor BI Prevents Nitric Oxide–Induced Cytotoxicity and Endotoxin-Induced Death

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Abstract

Nitric oxide (NO)-induced oxidative stress contributes to a variety of diseases. Although numerous mechanisms have been described controlling the production of NO, the mechanisms to prevent NO-induced cytotoxicity after NO synthesis are largely unknown. Here we report that scavenger receptor BI (SR-BI) prevents NO-induced cytotoxicity. Using CHO cell lines expressing wild-type and single-site mutant SR-BI protein, we demonstrate that SR-BI prevents NO-induced cytotoxicity and that a highly conserved CXXS redox motif is required for the anti-NO cytotoxicity activity of SR-BI. Using genetically manipulated mice, we demonstrate that SR-BI–null mice have a 3- to 4-fold increase in tyrosine nitrated proteins in aorta and liver compared with wild-type littermates, indicating that expression of SR-BI prevents peroxynitrite formation in vivo. Using lipopolysacharide (LPS)-challenged mice as an in vivo model of NO-induced cytotoxicity, we found that a single dose of LPS (120 000 U/g IP) induced 90% fatality of SR-BI–null mice within 3 days, whereas all of the wild-type littermates survived (n=20), demonstrating that SR-BI is highly protective against NO cytotoxicity in vivo. Importantly, SR-BI prevents LPS-induced death without eliminating NO production, suggesting that SR-BI prevents NO-induced cytotoxicity post-NO synthesis. Our study describes a novel observation that may shed new light on the treatment of nitric oxidative stress–associated diseases, such as septic shock.

Keywords

nitric oxide; scavenger receptor BI; nitric oxidative stress; endotoxin

Nitric oxide (NO) is a highly reactive molecule. NO reacts with superoxide to generate toxic peroxynitrite that causes cytotoxicity and tissue damage.[1] NO-induced oxidative

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stress (nitric oxidative stress) contributes to a variety of diseases, such as septic shock,[2] atherosclerosis,[3,4] Alzheimer's disease,[5,6] and diabetes.[7,8] NO is also an important signaling molecule. It functions to fight against infections,[2] regulate vascular tone,[3,4] and prevent platelet aggregation.[9] Therefore, how to prevent NO-induced cytotoxicity without eliminating NO production is a critical question. Although numerous mechanisms controlling the production of NO have been described[10] (for example, endothelial NO synthase [eNOS] activity is regulated by membrane targeting and by caveolin-1 binding),[10,11] mechanisms to prevent NO cytotoxicity after NO is generated are largely unknown.[12]

SR-BI is a membrane protein expressed in a variety of tissues.[13] SR-BI was originally described as an LDL receptor.[14] Subsequently, it was found that SR-BI is a physiological HDL receptor[15] and plays a key role in regulating plasma cholesterol levels.[16,17] Mice deficient in SR-BI have a 2-fold increase in cholesterol levels.[17] Using genetically manipulated mouse models, it was clearly demonstrated that SR-BI contributes to the development of cardiovascular diseases.[18–25] Recently, it has been demonstrated that SR-BI has additional functions besides regulating plasma cholesterol. SR-BI activates eNOS in endothelial cells[26,27] and induces apoptosis in the absence of HDL/eNOS.[28] In this study, we report that SR-BI prevents NO-induced cytotoxicity and endotoxin-induced death. Importantly, our study suggests that SR-BI suppresses NO-induced cytotoxicity without eliminating NO production.

Materials and Methods

The cytotoxicity detection method (lactate dehydrogenase [LDH] cytotoxicity assay kit) was from Roche (Indianapolis, Ind). The pLNCX2 vector was from Clontech Laboratories (Palo Alto, Calif). The anti–SR-BI serum was from Novus Biologicals (Littleton, Co). The monoclonal anti-nitrotyrosine antibody was from Calbiochem (La Jolla, Calif). Lipopolysacharide (LPS) (*Escherichia coli* serotype 055:B5; 3×10^6 units/mg, catalog no. L4005, lot no. 034k4112), sodium nitroprusside (SNP), DETA NONOate were from Sigma (St Louis, Mo).

Generation of Mutant Construct and CHO Cell Lines

Human SR-BI cDNA was provided by Dr Deneys van der West-huyzen (University of Kentucky). Mutant SR-BI (SRBI-C323G, cysteine-to-glycine mutant) was generated by PCR using wild-type SR-BI as the template and subcloned into pLNCX2 vector. The construct was transfected into CHO cells, and cell lines stably expressing the mutant SR-BI was obtained by G418 selection as we previously described.[26] The subcellular location of SRBI-C323G was determined by subcellular fraction combined with Western blot analysis using a method as previously described.[29] Similar to wild-type SR-BI, the SRBI-C323G mutant was localized in plasma membrane fraction, indicating the correct subcellular location of SRBI-C323G mutant (data not shown). CHO cell lines stably expressing vector (pLNCX2) or wild-type SR-BI was established as previously described.[26] The cells were cultured in Ham's F-12 medium containing 5% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.3 mg/mL G418.

NO-Induced Cytotoxicity Assays

Two methods were used to quantify NO-induced cytotoxicity. (1) LDH cytotoxicity assay, which quantitatively measures the LDH released by damaged cells, was performed using the cytotoxicity detection kit following the instructions. Data were expressed as percentage of the total LDH activity. (2) Single-clone assay was used to quantify the live cells. Briefly, CHO cells were cultured in complete medium to 80% confluence. The cells were then treated with 0.2 mmol/L or 0.4 mmol/L sodium nitroprusside (NO donor) for 24 hours in complete medium. Culture supernatant (100 μL) was removed for LDH assay, and the remaining cells were then trypsinized and placed in 10-cm dishes at 1:100 to 1:10 000 dilutions (to get single clones). Seven days later, cell clones appeared and were fixed with methanol and stained with Trypan blue. Each clone was derived from a single live cell. The cells were also treated with 0.1 mmol/L, 0.5 mmol/L, and 1 mmol/L of sodium cyanide to exclude the possibility that the cytotoxicity of SNP was caused by cyanide. No cytotoxicity was observed after 24 hours of sodium cyanide treatment (data not shown). The cells were also treated with 0.2 mmol/L, 0.3 mmol/L, or 0.4 mmol/L DETA NONOate for 40 hours in complete medium. The DETA NONOate–induced cytotoxicity was analyzed by LDH assay.

Western Blot

Cells or mouse tissues were lysed at 4°C for 20 minutes in lysis buffer consisting of 25 mmol/L 2-(N-morpholino)ethane sulfonic acid (MES), pH 6.5, 137 mmol/L NaCl, 1% (vol/vol) Triton X-100, 60 mmol/L octyl glucoside, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and 1% proteinase inhibitor cocktail (Sigma). After centrifugation (12 000 rpm, 5 minutes), the supernatant was mixed with one-fourth volume of reduced sample buffer and heated at 97°C for 5 minutes. Ten micrograms of protein were then separated by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, blotted with specific primary antibody, the peroxidase-conjugated secondary antibody, and detected by chemiluminescence.[26]

Animals

SR-BI$^{+/-}$ mice on a mixed C57BL/6×129 background were obtained from the Jackson Laboratory (Bar Harbor, Me). SR-BI–null mice and the wild-type littermates were generated by breeding SR-BI$^{+/-}$ mice. Mouse tail DNA was used for PCR genotyping following the method provided by the Jackson Laboratory. The animals were fed standard laboratory chow diet and housed under pathogen-free conditions. The female SR-BI–null mice are infertile. SR-BI–null mice have a 2-fold increase in plasma cholesterol levels compared with wild-type control mice.[17] Animal care and experiments were approved by the Institutional Animal Care and Use Committee.

Isolation of Aorta and Liver

The SR-BI–null mice and wild-type littermates (8 in each group) on chow diet were euthanized at the age of 10 to 12 weeks. After perfusion with PBS, the aorta and the liver were excised and homogenized in lysis buffer consisting of 25 mmol/L MES, pH 6.5, 137 mmol/L NaCl, 1% (vol/vol) Triton X-100, 60 mmol/L octyl glucoside, 0.1% (wt/vol) SDS, and 1% proteinase inhibitor cocktail.

Mouse Model of Endotoxin-Induced Death

A single dose of LPS (120 000 endotoxin units/g IP) was administered to 10- to 12-week-old SR-BI–null mice and wild-type littermates on chow diet (20 mice in each group). LPS was from the same lot number to exclude a batch effect. Survival was monitored for a 5-day period. Data are expressed as percentage of mice alive at each time point and analyzed by log-rank χ^2 test. It is important to point out that the lethal dosage of LPS varies with the age and genetic background of mice. To address this problem, we used only age-matched SR-BI–null and wild-type littermates that were generated by breeding SR-BI+/−mice.

Blood Chemistry and Histology

A single dose of LPS (120 000 endotoxin units/g IP) was administered to 10- to 12-week-old SR-BI–null mice (10 mice) and wild-type littermates (8 mice). Mice injected with PBS were used as controls (8 mice in each group). Twenty hours after LPS treatment, the mice that were still alive were euthanized by CO_2 inhalation. The blood was obtained by cardiac puncture. The serum nitrite/nitrate (NO_x) levels were measured with a nitrite/nitrate kit (Cayman, Ann harbor, Mich), and the serum chemistries were quantified by Division of Laboratory Animal Resources of the University of Kentucky using an Analyst Chemistry System. After perfusion with PBS, the liver were excised and fixed in 3% formaldehyde overnight. The tissues were embedded in paraffin and the sections were stained with hematoxylin/eosin.

Statistical Analysis

The survival assay was analyzed by log-rank χ^2 test using SAS software. Alternatively, paired or unpaired Student's t test was used. Means were considered different at $P<0.05$.

Results and Discussions

SR-BI Prevents NO-Induced Cytotoxicity

First, we used CHO cell lines stably expressing SR-BI to elucidate the effect of SR-BI on NO-induced cytotoxicity (Figure 1a). This CHO cell system has been widely used to study the functions of SR-BI, such as HDL cholesteryl ester uptake and eNOS activity.[15,26,27] We treated the cells with 0.2 mmol/L SNP and used phase-contrast micrography, a single-clone assay, and a cytotoxicity assay to elucidate the effect of SR-BI on NO-induced cytotoxicity. SNP is a NO donor widely used in NO cytotoxicity studies, both in vivo and in vitro.[30,31] After 24 hours of SNP treatment, most of the CHO-vector control cells underwent a morphological change consistent with cell death (Figure 1b). In contrast, all CHO-SRBI cells retained a healthy spindle-like morphology (Figure 1b), indicating that SR-BI protects cells from NO-induced cytotoxicity. When quantified by a single-clone assay, CHO-SRBI cells showed 10-fold more living cells compared with CHO-vector cells (Figure 1c). In agreement with the single-clone assay, a LDH cytotoxicity assay also showed that the expression of SR-BI significantly prevented NO-induced cytotoxicity (Figure 1d). We also treated the cells with 0.4 mmol/L SNP for 24 hours. As shown in Figure 1c and 1d, expression of SR-BI significantly suppressed NO-induced cytotoxicity. A potential problem in these studies is that SNP also releases cyanide.[32] We therefore treated CHO vector and

CHO-SRBI cells with 0.1 mmol/L, 0.5 mmol/L, and 1 mmol/L sodium cyanide for 24 hours to determine whether the release of cyanide affected cell viability. No cytotoxicity was observed, which suggests that cyanide was not responsible for the cell death (Data not shown).

A CXXS Motif Is required for the Anti-NO–Induced Cytotoxicity Activity of SR-BI

Next, we explored the molecular mechanism underlying SR-BI–mediated protection against NO-induced cytotoxicity. A motif analysis revealed that SR-BI contains a highly conserved putative redox motif at C323XXS326 (Figure 2a). The CXXS motif belongs to the CXXC redox protein family and plays important roles in cellular redox regulation and antioxidant defense.[33] To determine whether this putative redox motif was responsible for the ability of SR-BI to protect against NO-induced cell damage, we generated a CHO cell line stably expressing C323-to-G323 mutant SR-BI (CHO-SRBI-C323G mutant) (Figure 2b). Morphological and quantitative assays (Figure 2c through 2e) showed that this mutant SR-BI completely lost its ability to protect NO-induced cell damage, indicating that the CXXS redox motif is required for the anti-NO cytotoxicity activity of SR-BI.

SNP releases a NO moiety in a complex mechanism. SR-BI may affect the metabolism of SNP or the release of NO from SNP. Therefore, we tested another NO donor (DETA NONOate, which releases NO in a kinetically controlled manner) to confirm that SR-BI prevents NO-induced cytotoxicity. As shown in Figure 2f, LDH cytotoxicity assay showed that the expression of SR-BI significantly suppresses DETA NONOate–induced cytotoxicity, whereas SRBI-C323G mutant failed to prevent DETA NONOate–induced cytotoxicity.

SR-BI Suppresses Nitric Oxidative Stress in Aorta and Liver

Then, we tested whether the expression of SR-BI can suppress the extent of NO-induced oxidative stress in vivo. NO reacts with superoxide to produce the highly toxic and potent oxidant peroxynitrite. Peroxynitrite then reacts with the tyrosine residues of proteins to generate tyrosine nitrated proteins (nitrotyrosine). The formation of these nitrotyrosine residues can disrupt protein function and cause cytotoxicity.[1] Tyrosine-nitrated proteins are considered a fingerprint of nitric oxidative stress in vivo and a major 50-kDa nitrotyrosine band can be detected by a commercial anti-nitrotyrosine antibody.[34] Therefore, we quantified the extent of tyrosine nitrated proteins in mouse aorta isolated from SR-BI–null mice and wild-type littermates. Western blot analysis showed that the aorta of SR-BI–null mice had a 3-fold increase in tyrosine-nitrated proteins compared with wild-type mice, indicating that SR-BI is capable of preventing protein tyrosine nitration in vivo (Figure 3a and 3c). We also measured the extent of tyrosine nitrated proteins in the liver (another tissue that expresses SR-BI). Expression of SR-BI dramatically suppressed protein tyrosine nitration in the liver (Figure 3b and 3d).

SR-BI Prevents Endotoxin-Induced Death

Finally, we used endotoxin-challenged mice, an in vivo model of NO-induced cytotoxicity, to test whether SR-BI can suppress NO-induced cytotoxicity at the whole animal level. A single dose of LPS (120 000 U/g IP) to SR-BI–null mice and wild-type littermates (10 to 12 weeks of age, 20 mice in each group) induced 20%, 80%, and 90% fatality of SR-BI–

null mice at 1, 2, and 3 days of LPS treatment, respectively. In contrast to SR-BI–null mice, all of the wild-type littermates survived, demonstrating that SR-BI is highly protective against LPS-induced death (Figure 4a). SR-BI–mediated protection against LPS-induced liver injury was assessed by histology and by measuring serum alanine aminotransferase (ALT) levels. Compared with wild-type controls, SR-BI–null mice exhibited significant cell death in the liver (Figure 4b, arrows indicate necrotic cells) and had a dramatic increase in serum ALT levels (Figure 4c) 20 hours following LPS treatment. NO plays a key role in endotoxin-induced death.[2] We therefore measured serum nitrite and nitrate (NO_x) levels to estimate NO production in vivo. The serum NOx levels were dramatically increased in both SR-BI–null and wild-type mice on LPS treatment (Figure 4d). However, NO_x levels in SR-BI–null mice were only moderately increased compared with wild-type mice (Figure 4d), indicating that expression of SR-BI does not eliminate NO production. These data suggest that expression of SR-BI prevents NO-induced cytotoxicity not by eliminating NO production, rather by a mechanism that has not yet been identified.

It has been reported that all classes of lipoproteins (VLDL, LDL, and HDL) bind to LPS and neutralize the toxicity of LPS.[35] The protective effect of lipoproteins on LPS-induced death is demonstrated by the report of Netea et al that mice deficient in LDL receptor have an increase in lipoprotein levels and are more resistant to LPS-induced death.[36] In contrast, our study shows that mice deficient in SR-BI have an increase in lipoprotein levels but are more susceptible to LPS-induced death compared with wild-type mice. Therefore, an increase in lipoprotein levels in SR-BI–null mice is not likely the mechanism responsible for the ability of SR-BI to protect against LPS-induced death. In this study, we demonstrate that SR-BI significantly suppresses tyrosine-nitrated protein formation and prevents NO-induced cytotoxicity. These data suggest that SR-BI prevents endotoxin-induced animal death via suppressing NO-induced cytotoxicity. Further study is needed to determine whether SR-BI protection against NO cytotoxicity is secondary/indirect consequences of SR-BI–dependent effects on cellular lipid metabolism. For example, expression of SR-BI may change the lipid components of the plasma membrane, which then affects membrane-mediated signaling.

Septic shock is the 13th cause of death that claims more than 215 000 lives per year in the Unites States.[37,38] In septic shock, massive infections stimulate inducible NOS to generate high levels of NO, which causes cytotoxicity and tissue damage.[2] In the early 1990s, it was expected that inhibition of NO production would prevent septic shock. However, animal and clinical studies have proven that simply inhibiting NO production by a NOS inhibitor is not an efficient way to prevent septic shock.[39,40] The reason for this lack of protection is that NO has both beneficial and deleterious effects.[1] NO is required to fight against infections,[2] regulate vascular tone,[3,4] and prevent platelet aggregation.[9] Therefore, an ideal target for the treatment of septic shock is to prevent NO-induced cytotoxicity after NO synthesis, not to eliminate NO production. SR-BI is a likely candidate to fulfill the task. In this study, we demonstrate that SR-BI, a known stimulator of eNOS,[26,27] effectively prevents endotoxin-induced death. Importantly, our study suggests that SR-BI suppresses NO-induced cytotoxicity without eliminating NO production. This special feature of SR-BI may shed new light on the treatment of septic shock via an anti-NO cytotoxicity mechanism.

In summary, we used 3 different systems to demonstrate that SR-BI is highly protective against NO-induced cytotoxicity. In addition, we demonstrate that a putative redox motif in SR-BI is required for protection from NO-induced cytotoxicity. These findings may shed new light on the treatment of nitric oxidative stress-associated diseases, such as septic shock.

Acknowledgments

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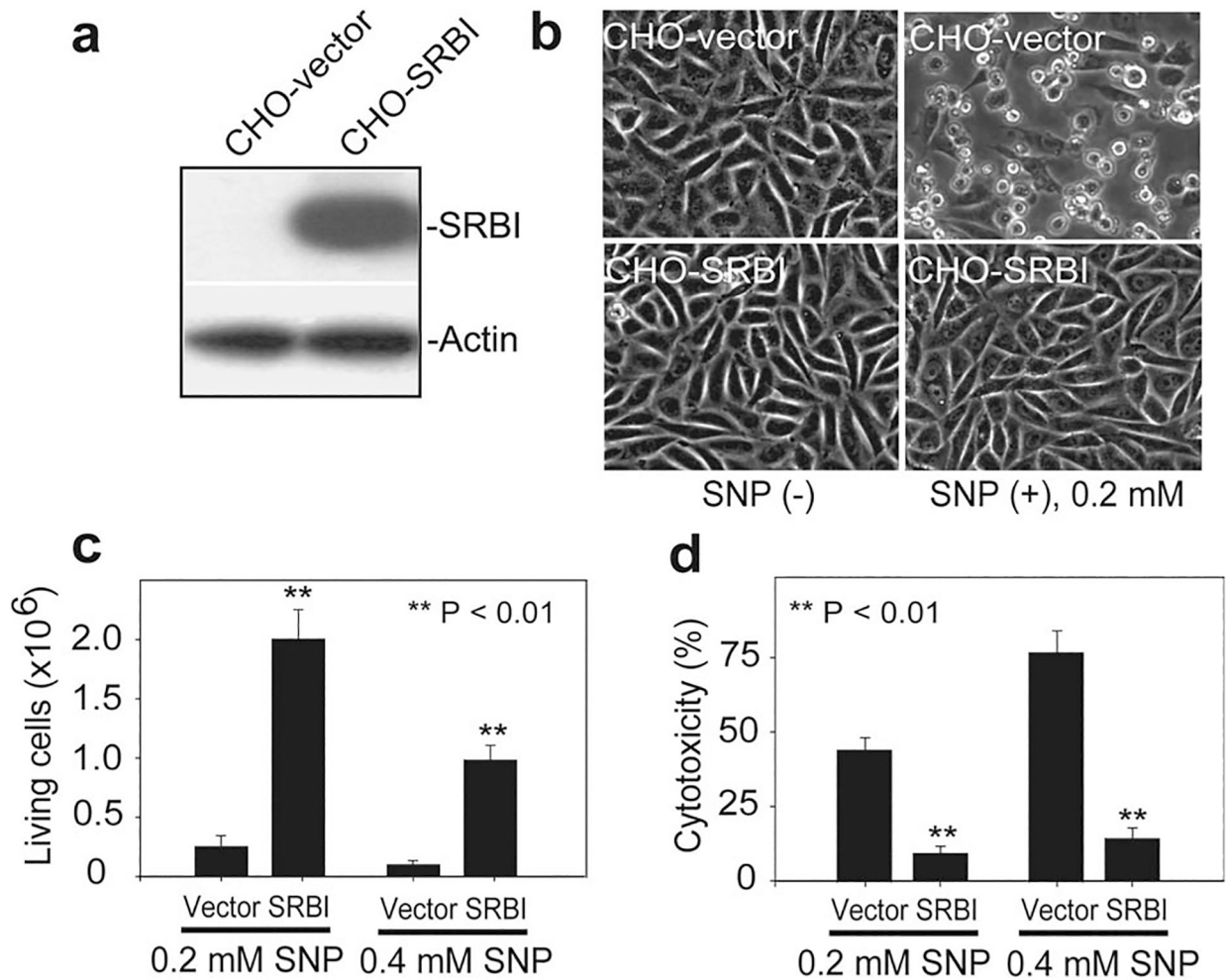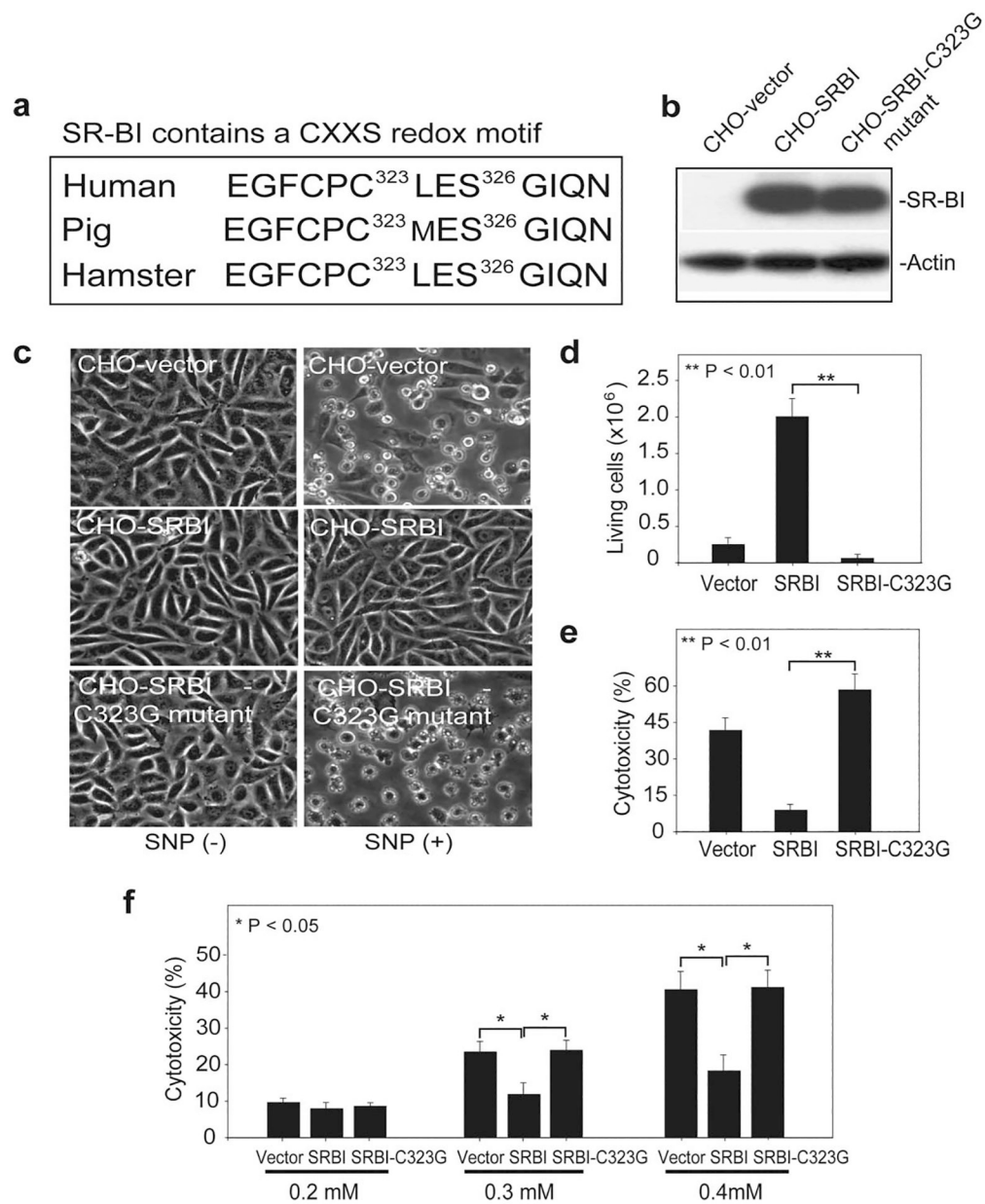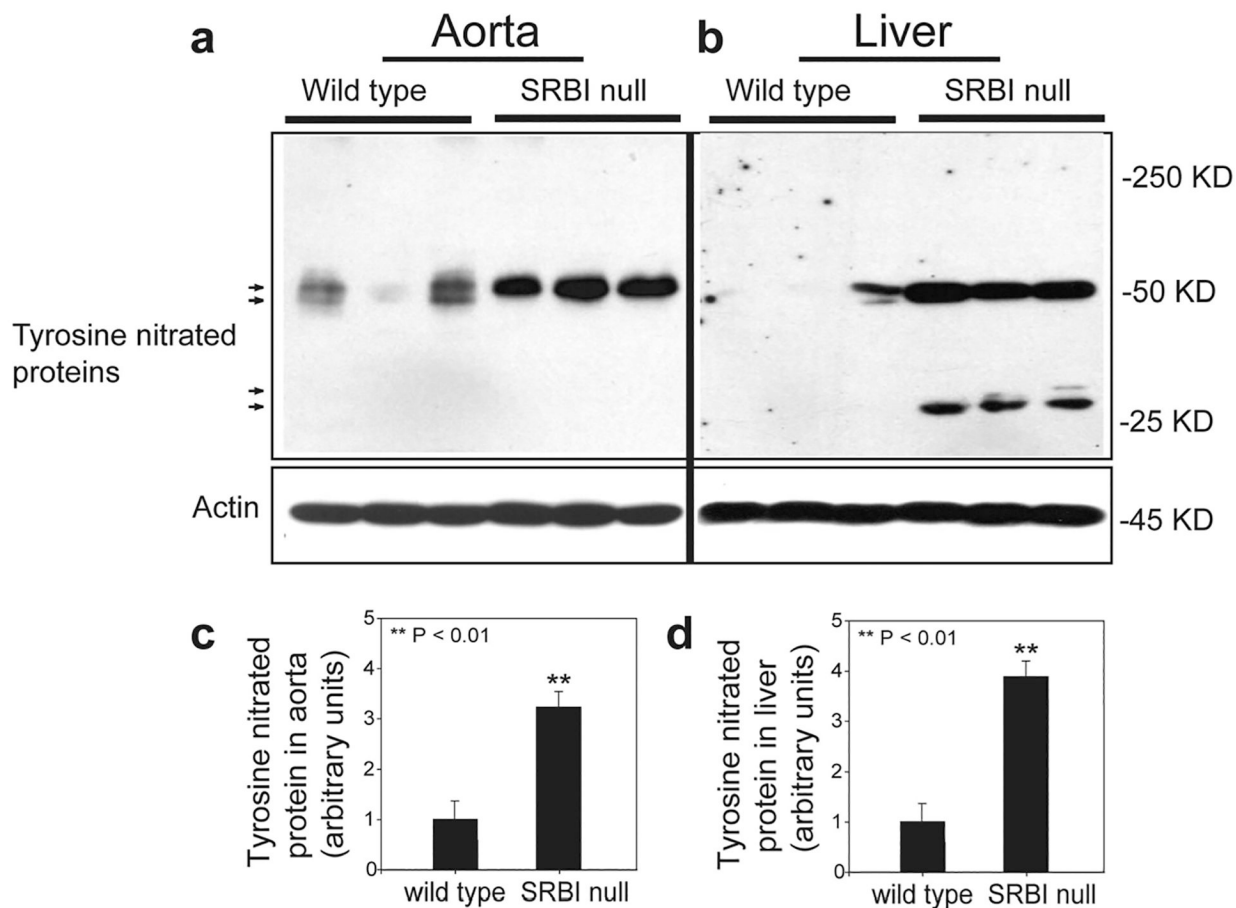
Figure 1.
SR-BI prevents NO-induced cytotoxicity. CHO cells stably expressing SR-BI (CHO-SRBI) or vector control were grown in complete medium to 80% confluence. Expression of SR-BI was confirmed by Western blot analysis (a). The cells were treated with 0.2 mmol/L or 0.4 mmol/L SNP in complete medium for 24 hours. Cells without SNP treatment were used as control. The cytotoxicity was analyzed by phase-contrast micrography ($\times 10$) (b), by single-clone assay to quantify cell survival (c), and by LDH cytotoxicity assay to quantify cell damage (d). All of the assays showed that expression of SR-BI significantly suppressed NO-induced cytotoxicity. **P<0.01 vs CHO-vector. The data are from 3 independent experiments.

Figure 2.
A CXXS motif is required for the anti-NO–induced cytotoxicity activity of SR-BI. SR-BI contains a highly conserved CXXS motif (a). CHO cells stably expressing vector, wild-type SR-BI (CHO-SRBI), or mutant SR-BI (CHO-SRBI-C323G) were grown in complete medium to 80% confluence. The expression of mutant SR-BI was confirmed by Western blot analysis using an antibody against SR-BI (b). The cells were treated with 0.2 mmol/L SNP for 24 hours. The SNP-induced cytotoxicity was analyzed by phase contrast micrograph (×10) (c), by single-clone assay to quantify survival cells (d), and by LDH cytotoxicity assay (e). All of the assays showed that the SRBI-C323G mutant lost completely the ability of SR-BI against NO-induced cytotoxicity. **$P<0.01$ vs CHO-SRBI. The cells were also treated with 0.2 mmol/L, 0.3 mmol/L, or 0.4 mmol/L DETA NONOate for 40 hours,

and the cytotoxicity was analyzed by measuring LDH activity in the culture supernatant (f). Expression of SR-BI significantly suppressed DETA NONOate–induced cytotoxicity (*P<0.05 vs CHO-vector), whereas SRBI-C323G mutant lost completely the ability of SR-BI against DETA NONOate–induced cytotoxicity (*P<0.05 vs CHO-SRBI). The data are from 3 independent experiments.

Figure 3.
SR-BI prevents tyrosine nitrated protein formation in mouse aorta and liver. SR-BI–null mice and wild-type littermates on chow diet were euthanized at the age of 10 to 12 weeks. After perfusion with PBS, the aorta (a and c) and the liver (b and d) were excised and homogenized in lysis buffer. Ten micrograms of protein were applied to a 10% SDS-PAGE under reducing conditions. The tyrosine nitrated protein was detected by Western blot analysis using a specific antibody against tyrosine nitrated protein (a and b, representative blot). Actin was used as loading control. The tyrosine nitrated protein bands were quantitatively analyzed by scanning densitometry and normalized to actin level (c and d) (n=8 per group). **P<0.01 vs wild type. A nonimmune mouse IgG was used as negative control to test the specificity of the antibody. No positive band was observed (data not shown).
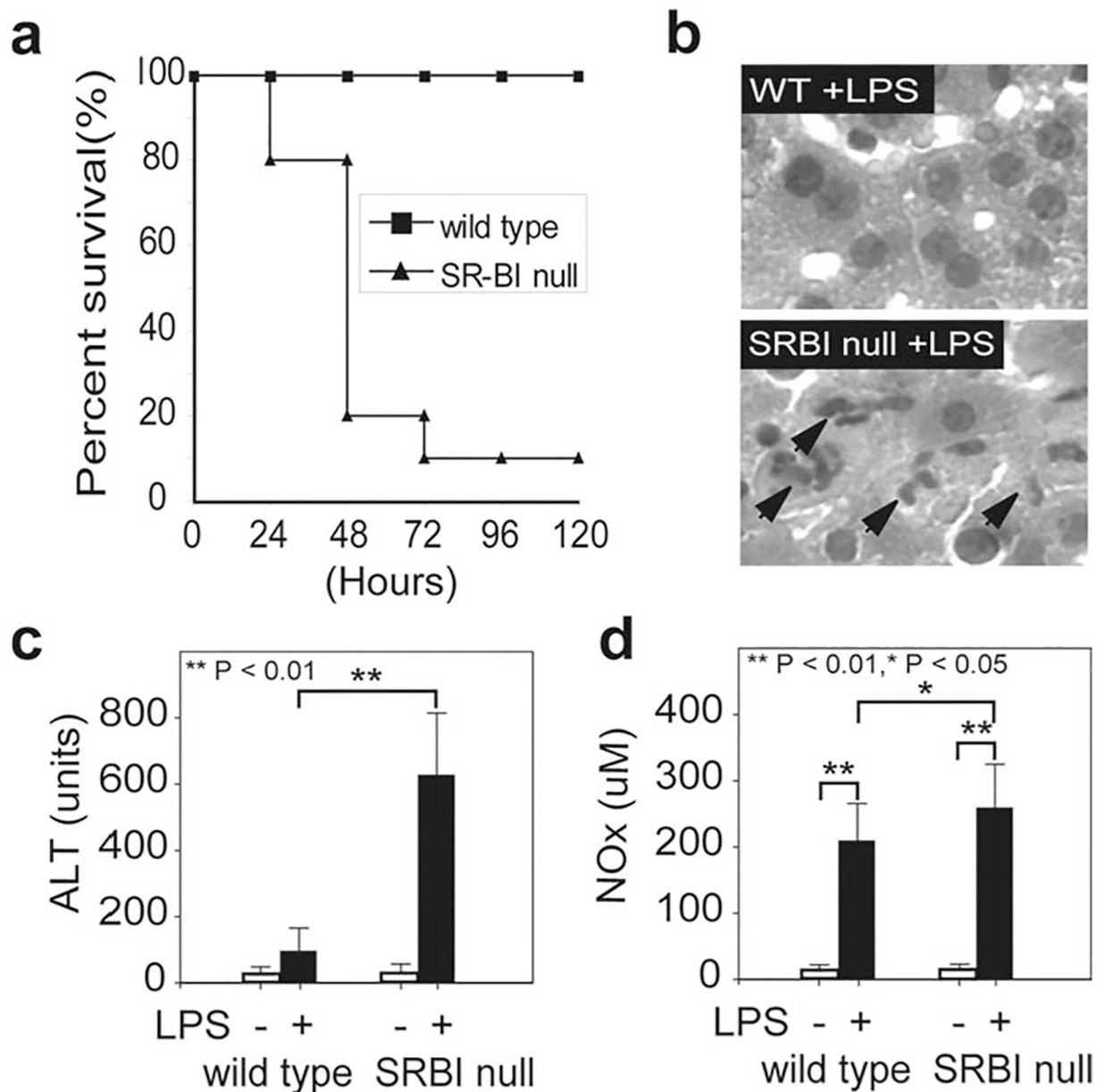
Figure 4.
SR-BI prevents LPS-induced death. a, Survival of LPS-challenged mice. A single dose of LPS (120 000 U/g IP) was administered to 10- to 12-week-old SR-BI–null mice (triangle) and wild-type littermates (square). Survival of SR-BI–null mice was significantly lower than that of wild-type mice. n=20 per group; $P<0.001$ vs wild type. b, Histology of LPS-challenged mice. Twenty hours following LPS injection, SR-BI–null mice exhibited significant cell death in the liver (arrows indicate necrotic cells). c, The serum marker of liver damage in LPS-challenged mice. Liver injury was assessed by measurement of serum levels of ALT. Twenty hours following LPS injection, ALT level was significantly higher in SR-BI–null mice (filled) than in wild-type controls (filled). n=8 per group; $**P<0.01$ vs wild type. d, Serum nitrite/nitrate (NO_x) levels in LPS-challenged mice. Twenty hours following

LPS injection, the serum NO_x levels were dramatically increased in both SR-BI–null and wild-type mice (filled) compared with PBS-treated control mice (open). n=8 per group; **P<0.01 vs PBS-treated control mice. However, NO_x levels in SR-BI–null mice (filled) were only moderately increased compared with wild-type mice (filled) on LPS treatment. n=8 per group; *P<0.05 vs LPS-treated wild-type mice.