SR-BI protects against endotoxemia in mice through its roles in glucocorticoid production and hepatic clearance

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Septic shock results from an uncontrolled inflammatory response, mediated primarily by LPS. Cholesterol transport plays an important role in the host response to LPS, as LPS is neutralized by lipoproteins and adrenal cholesterol uptake is required for antiinflamatory glucocorticoid synthesis. In this study, we show that scavenger receptor B-I (SR-BI), an HDL receptor that mediates HDL cholesterol ester uptake into cells, is required for the normal antiinflammatory response to LPS-induced endotoxic shock. Despite elevated plasma HDL levels, SR-BI–null mice displayed an uncontrollable inflammatory cytokine response and a markedly higher lethality rate than control mice in response to LPS. In addition, SR-BI–null mice showed a lack of inducible glucocorticoid synthesis in response to LPS, bacterial infection, stress, or ACTH. Glucocorticoid insufficiency in SR-BI–null mice was due to primary adrenal malfunction resulting from deficient cholesterol delivery from HDL. Furthermore, corticosterone supplementation decreased the sensitivity of SR-BI–null mice to LPS. Plasma from control and SR-BI–null mice exhibited a similar ability to neutralize LPS, whereas SR-BI–null mice showed decreased plasma clearance of LPS into the liver and hepatocytes compared with normal mice. We conclude that SR-BI in mice is required for the antiinflammatory response to LPS-induced endotoxic shock, likely through its essential role in facilitating glucocorticoid production and LPS hepatic clearance.

Introduction
Endotoxic shock remains a significant cause of death despite the use of various therapeutic approaches (1). LPS is the primary cause of Gram-negative sepsis. LPS, through its interaction with the LPS-binding protein–containing (LBP-containing) LBP-CD14-TLR4 complex, activates macrophages, causing the release of inflammatory cytokines (2). While the inflammatory response represents a host defense to invading pathogens, uncontrolled systemic inflammation can lead to serious systemic complications such as disseminated intravascular coagulation, tissue damage, and endotoxic shock (2).

LPS can be neutralized by the major lipoproteins, HDL, LDL, VLDL, and chylomicrons as well as apolipoproteins apoE and apoA-I (3–5). apoE may protect by redirecting LPS from Kupffer cells to hepatocytes (3). The preferential binding of LBP to HDL accounts for HDL being the primary carrier of LPS in the blood (6). While LPS is cleared mainly by hepatocytes, the mechanism or mechanisms are unclear. Although scavenger receptor A (SR-A) has been reported to mediate LPS uptake and detoxification (7), SR-A–null mice showed unchanged production of TNF-α in response to LPS (8), casting doubt on the physiological role of SR-A in sepsis. These findings suggest the involvement of other scavenger receptors, such as scavenger receptor B-I (SR-BI), in LPS metabolism.

SR-BI is an HDL receptor that plays a key role in the reverse cholesterol transport pathway through mediating selective lipid uptake from HDL particles (9, 10). SR-BI in the liver is highly expressed in hepatocytes (9) and to a lesser extent in Kupffer cells (11) and functions to clear HDL cholesterol into the bile (12). Studies using SR-BI–deficient and –transgenic mice have shown that SR-BI is protective against atherosclerosis (10). SR-BI has a broad ligand-binding specificity and is involved in the binding and cellular uptake of various endogenous ligands, such as β-amylloid and serum amyloid A (13), apoptotic cells, and exogenous pathogens (10). For example, SR-BI plays a role as a receptor for hepatitis C virus and is required for virus infection (14). Importantly, SR-BI also mediates the binding and uptake of LPS (15), the major Gram-negative bacterial cell wall component, in macrophages and HeLa cells overexpressing SR-BI, and lipoteichoic acid (LTA) (16), a component of Gram-positive bacteria. Furthermore, SR-BI and its alternatively spliced variant, SR-BII (17), have been shown to directly bind a variety of bacteria with little discrimination, suggesting a conserved role for these receptors in pattern recognition and innate immunity (18). This is supported by the fact that SR-BI–null mice are more susceptible to LPS-induced death, a finding attributed to a protective effect of SR-BI in inhibiting NO-induced cell toxicity (19). A recent study, however, reported that SR-BI facilitates bacterial invasion and proliferation in cells, with evasion of lysosomal processing (16), indicating that SR-BI might facilitate bacterial infection and sepsis.

SR-BI is highly expressed in the adrenals, ovary, and testis, where it functions to provide cholesterol for steroid hormone synthesis through the selective cholesterol ester (CE) uptake from HDL and LDL (20–22). Although the LDL receptor (LDLR) also contributes
to cholesterol delivery through LDL uptake (23), SR-BI–mediated HDL cholesterol uptake appears to be the major source of cholesterol for glucocorticoid synthesis in rodents (20, 24, 25). A critical role of SR-BI in humans is suggested by the observation that patients with familial hypercholesterolemia that lack functional LDLR show a normal response to ACTH (26). Glucocorticoids exert antiinflammatory effects by suppressing proinflammatory cytokines (e.g. TNF-α, IL-1, IL-6) and stimulating antiinflammatory mediators (IL-10, TGF-β) (27). Adrenal deficiency, characterized by glucocorticoid insufficiency, is often associated with sepsis (28). Common causes of adrenal failure are impaired function of the upstream hypothalamic-pituitary-adrenal axis (secondary adrenal insufficiency) and malfunction or destruction of the adrenal glands (primary adrenal insufficiency). Since only small amounts of glucocorticoids are stored in the adrenals (29), primary adrenal failure in endotoxic shock is usually due to insufficient glucocorticoid biosynthesis (29). Depletion of plasma HDL in apoA-I–null mice (30) or by inactivation of SR-BI (31) markedly reduces CE content in the adrenals. In the case of apoA-I–null mice, adrenal function was affected and corticosterone production was limited (30). In SR-BI–null mice, adrenal function appeared unaffected under normal physiological conditions (10).

In this study, we have investigated the role of SR-BI in regulating LPS-induced inflammation and endotoxic shock. Studies using control and SR-BI–null mice have shown that SR-BI expression is essential for stimulated glucocorticoid production in the adrenals in response to LPS and stress, and that glucocorticoid insufficiency as well as reduced LPS clearance likely contribute to the enhanced inflammation and endotoxin-induced death in SR-BI–null mice.

Results

SR-BI–null mice show an exaggerated inflammatory response to LPS-induced endotoxic shock. To determine the influence of SR-BI on LPS-induced inflammation, SR-BI–null and control mice were injected i.p. with LPS (0.5 μg/g body weight), and serum cytokine levels were determined by ELISA at various times following injection. As shown in Figure 1, LPS resulted in a strong induction of both TNF-α and IL-6. In the case of TNF-α, maximum concentrations were reached by 2 hours and were markedly higher in the SR-BI–null mice than in control mice (Figure 1A). In the case of IL-6, LPS-induced plasma concentrations reached nearly similar maximal levels in both SR-BI–null and control mice after 2 hours (Figure 1B). However, IL-6 levels in control mice returned to baseline levels after 8 hours, whereas the level of IL-6 in SR-BI–null mice remained elevated for 24 hours before dropping to baseline levels by 48 hours. An increased TNF-α and IL-6 cytokine response in SR-BI–null mice compared with control mice was evident at each LPS concentration tested, and no cytokine induction was observed in mice injected with saline alone (data not shown). TNF-α and IL-6 expression in the livers of these mice were assessed by quantitative real-time PCR analysis (Q-PCR) of mRNA levels. In line with the observed differences in protein expression, SR-BI–null mice had higher expression of liver mRNA for both TNF-α and IL-6 in response to LPS than control mice (Figure 1, C and D). However, the lack of close correlation between liver mRNA levels and plasma cytokine levels suggests posttranscriptional regulation mechanisms and/or the contribution of nonhepatic cytokine production.

A cytokine array analysis of plasma at either 2 or 24 hours after LPS injection showed that the levels of a variety of proinflammatory cytokines were increased to a significantly greater extent in SR-BI–null mice compared with control mice (Table 1). Cytokines that showed no difference between SR-BI–null and control mice at 2 hours are also listed in Table 1. Of these, IL-5, IL-6, and IL-10 levels were significantly greater in SR-BI–null mice at 24 hours, whereas the remainder showed no difference at 24 hours (data not shown). Of all the cytokines assayed, IL-4, IL-7, IL-13, and IL-15 did not show any response to LPS in either of the 2 genotypes. These are Th2 cytokines associated with allergy, autoimmune disease, and parasitic infection. Under basal conditions in the absence of LPS, no differences in cytokine levels were observed between SR-BI–null and control mice. These data clearly show that SR-BI–null mice exhibit a markedly increased inflammatory response to LPS compared with control SR-BI+/+ mice.

An overproduction of proinflammatory cytokines caused by LPS in SR-BI–null mice suggests increased susceptibility to endotoxic shock and death in these animals. Consistent with this hypothesis, we observed a marked difference in survival rate of SR-BI–null and control mice injected i.p. with a single relatively moderate dose of LPS (5 μg/g body weight) (Figure 2). Neither the control group
nor the SR-BI heterozygous (+/-) group had any fatalities (n = 8), whereas there was a 100% mortality rate within 3 days in the SR-BI–null group (n = 8). The SR-BI–null mice showed symptoms of stress, including reduced movement, shivering, and diarrhea, whereas both the control mice and heterozygous mice appeared free of these symptoms. As was the case for mice treated with 0.5 μg/g body weight LPS (Figure 1 and Table 1), SR-BI–null mice treated with the higher dose of LPS (5 μg/g body weight) showed markedly enhanced plasma levels of TNF-α at 2 hours compared with the control mice, whereas IL-6 levels were induced to the same extent in the 2 mouse groups at the same time point (Figure 2B).
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Adrenal insufficiency contributes to an enhanced inflammatory response and endotoxic shock in SR-BI–null mice. To further investigate the role of the adrenals in the LPS-induced inflammatory response, both control and SR-BI–null mice were adrenalectomized and then treated 7 days after recovery with LPS. It should be noted that adrenalectomy renders mice deficient, not only in corticosterone, but also in other adrenal hormones that may each have a role in inflammation (33). LPS was used at a relatively low dose (0.125 µg/g body weight) since adrenalectomy increases sensitivity in mice to inflammation (34). Prior to LPS injection, basal levels of both TNF-α and MCP-1 were similarly low in control and SR-BI–null mice (data not shown). In the sham-operated SR-BI–null mice, there was an inflammatory response to LPS such that the levels of TNF-α, IL-6, and MCP-1 were markedly greater than the corresponding levels in control SR-BI+/+ mice 2 hours following LPS injection (Figure 6, A–C). A greater inflammatory response to LPS was observed in the adrenalectomized mice from both groups. In the case of TNF-α, levels were approximately 38% higher (P < 0.05) in the adrenalectomized SR-BI–null mice than in control mice (Figure 6A). However, no differences in either IL-6 or MCP-1 levels were observed between adrenalectomized SR-BI–null and control mice under conditions in which the levels of the 2 cytokines were both extremely high (Figure 6, B and C). The differences observed in IL-6 and MCP-1 levels between sham-operated control and SR-BI–null mice treated with LPS were not observed in the experiments shown in Figure 1B and Table 1. This difference is likely related to the lower dose of LPS used in the experiment shown in Figure 6. Thus, while cytokine levels in

Figure 2
SR-BI+/+ mice are more sensitive to LPS-induced lethality. (A) Survival of SR-BI+/+ mice in response to LPS. SR-BI–/–, SR-BI+/–, and SR-BI+/+ mice (n = 8) were injected i.p. with 5 µg/g body weight of LPS. Mice were carefully monitored, and survival rates at the indicated times were recorded (SR-BI+/+ vs. SR-BI–/–, P < 0.001). (B) Plasma cytokine levels in SR-BI–/– and SR-BI+/+ mice challenged with LPS. LPS (5 µg/g body weight) was injected i.p. into SR-BI–/– and SR-BI+/+ mice, and blood was collected 2 hours following LPS injection. Plasma IL-6 and TNF-α levels were determined by ELISA. Values shown are the mean ± SD (n = 4) (*P < 0.05; **P < 0.01). Similar results were seen in 3 independent experiments.
SR-BI–null mice reached similar and possibly maximal levels at the 2 LPS doses used, the responses of IL-6 and MCP-1 in control mice were much lower at the lower LPS dose. These results are in line with an enhanced sensitivity of SR-BI–null mice to low doses of LPS. Overall, the results of this experiment show that adrenal-ectomy to a large extent abolished the differences in inflammatory cytokines between SR-BI–null and control mice.

LPS-treated adrenalectomized mice of both control and SR-BI–null groups showed low levels of plasma corticosterone that were similar to the levels in untreated SR-BI–null mice (Figure 6D). These data further confirm the finding that a lack of SR-BI prevents LPS-induced production of corticosterone in adrenals. In adrenalectomized mice, the levels of corticosterone were low but significant and were higher in control than in SR-BI–null mice. Extra-adrenal glucocorticoid synthesis has been reported (33), and our results point to the possibility that such extra-adrenal synthesis may also be dependent on SR-BI. Together, these data suggest that adrenal insufficiency plays a major role in contributing to the enhanced LPS-induced inflammatory response in SR-BI–null mice. At the same time, the significant difference between TNF-α levels in adrenalectomized SR-BI–null and control mice supports a conclusion that SR-BI influences inflammation by mechanisms in addition to its role on adrenal glucocorticoid production.

To address the possible mechanisms for adrenal insufficiency in SR-BI–null mice, we first examined ERK phosphorylation, a key step in the ACTH-induced signaling pathway in the adrenals (35). ERK phosphorylation to p-ERK was found to be induced by LPS within 1 hour in adrenals to a similar extent in SR-BI–null mice and control mice, with no significant changes in the levels of total ERK during the same period (data not shown). This result is consistent with ACTH signaling in the adrenals being initiated in a normal manner by ACTH in SR-BI–null mice. Two key enzymes in the biosynthesis of glucocorticoids that are transcriptionally induced during stimulation of corticosterone production from cholesterol are steroidogenic acute regulatory protein (StAR) and cytochrome P450 side-chain cleavage enzyme (Cyp11A1) (29). StAR is required for cholesterol transport across the inner mitochondrial membrane, and Cyp11A1 converts cholesterol into pregnenolone by catalyzing cholesterol side-chain cleavage. In untreated mice, the expression of both StAR and Cyp11A1 mRNA in adrenals was similar in SR-BI–null and control mice, as assessed by adrenal mRNA levels (Figure 7, A and B). Expression of mRNA for both proteins was upregulated by LPS within 1 hour in control mice and to at least the same extent in SR-BI–null mice. mRNA levels for both proteins remained higher in SR-BI–null mice compared with control mice for at least 24 hours. In line with StAR transcriptional
upregulation, the levels of StAR protein in the adrenals were also upregulated in both groups of mice and at least to the same extent in SR-BI-null mice as in control mice (Figure 7C). These results again suggest normal adrenal regulatory responses in respect to ACTH signaling in SR-BI-null mice.

The availability of unesterified cellular cholesterol is a key determinant in corticosterone production (29). To assess whether the inability of SR-BI–null mice to induce corticosterone synthesis in SR-BI–null mice results from limited cholesterol availability in the adrenal, we analyzed the expression of 2 proteins, the LDLR and HMG-CoA reductase, whose expression at the transcriptional level is highly regulated by the level of intracellular cholesterol (25, 36). The LDLR serves to internalize LDL, and HMG-CoA reductase is the key regulatory enzyme in cholesterol biosynthesis. Adrenal mRNA levels for the 2 proteins were determined in control and SR-BI–null mice before and after LPS treatment (Figure 7, D and E). Basal LDLR mRNA levels were about 2-fold greater in SR-BI–null than in control mice, indicative of decreased cellular cholesterol levels in SR-BI–null mice, as previously reported by others (31) (Figure 7D). In the case of control mice, mRNA levels for LDLR were moderately increased (<2 fold) following LPS treatment and returned to unstimulated levels within 24 hours. In contrast, LPS induced a striking increase in LDLR mRNA levels in SR-BI–null mice to levels that were about 4- to 5-fold higher than those in control mice. Similarly, HMG-CoA reductase was markedly upregulated (about 7-fold) in SR-BI–null mice but was relatively unchanged in control mice following LPS treatment (Figure 7E). The extensive transcriptional upregulation of these

Figure 6
Effects of adrenalectomy on LPS-induced response in SR-BI+/+ and SR-BI–/– mice. Mice were adrenalectomized and allowed to recover for 1 week. Adrenalectomized mice (ADX) and sham-operated mice (sham) were injected i.p. with LPS (0.125 μg/g body weight), and 2 hours later, plasma cytokines and corticosterone levels were determined. (A) Plasma TNF-α levels. (B) Plasma IL-6 levels. (C) Plasma MCP-1 levels. (D) Plasma corticosterone levels. Values shown are mean ± SD (n = 5). *P < 0.05; **P < 0.01. Similar results were found in 3 independent experiments.

Figure 7
Adrenal gene expression during LPS-induced inflammatory response. SR-BI+/+ and SR-BI–/– mice were injected i.p. with 0.5 μg/g body weight of LPS. Mice were sacrificed at indicated time points, and adrenals were collected for mRNA extraction and Q-PCR. (A) Adrenal STAR mRNA. (B) Adrenal Cyp11A1 mRNA. (C) Adrenal STAR Western blot (10 μg cell protein/lane). Mice were sacrificed 2 hours after LPS injection. Adrenals were collected, and proteins were extracted. (D) Adrenal LDLR mRNA. (E) Adrenal HMG-CoA reductase mRNA. Values shown are mean ± SD (n = 3) of the ratio of genes for the target genes to that of the 18S rRNA. Similar results were found in 2 independent experiments.
2 proteins by LPS in SR-BI–null mice indicates that, following LPS treatment, there is a rapid and marked decrease in cellular unesterified cholesterol, presumably due to an inability of cells to replenish intracellular cholesterol from circulating lipoproteins, intracellular biosynthesis, or cellular CE stores. Recent studies suggest that the cholesterol transporter ABCA1 may also contribute to adrenal cholesterol regulation (37). We evaluated adrenal ABCA1 expression in mice in response to LPS and found that both ABCA1 mRNA and protein levels were significantly lower in SR-BI–null mice, suggesting that ABCA1 did not contribute to low cellular cholesterol levels in these mice (data not shown).

We conclude from these results that SR-BI in the adrenals is required to provide, through the process of selective CE uptake, sufficient cholesterol for glucocorticoid production under conditions of high adrenal stimulation by ACTH, such as occurs during LPS-induced inflammation.

SR-BI plays a role in LPS clearance into hepatocytes. Since LPS is cleared largely via hepatocytes (8), which express high levels of SR-BI, and since SR-BI functions as a receptor for both HDL and LPS, we investigated whether SR-BI is responsible for LPS clearance by the liver. Cultured primary hepatocytes were isolated from control and SR-BI–null mice and assayed for their ability to take up LPS. As shown in Figure 8A, fluorescently labeled Alexa Fluor–LPS was taken up rapidly within 1 hour into control hepatocytes but to a significantly lesser extent in SR-BI–null hepatocytes. Similarly, cell association of 125I-LPS was decreased in SR-BI–null hepatocytes (Figure 8B). Interestingly, SR-BI can mediate 125I-LPS uptake more efficiently when LPS is in its HDL-associated form. While the uptake of LPS is reduced in SR-BI–null hepatocytes, a significant level of SR-BI–independent uptake was observed, suggesting the involvement of other candidate receptors, such as SR-A, in this process. The clearance of 125I-LPS from plasma and its uptake into the liver was also investigated in control and SR-BI–null mice. First, the uptake of 125I-LPS into hepatocytes and nonhepatocytes isolated from livers collected 2 hours after LPS injection was measured. In line with the uptake of Alexa Fluor–LPS in cultured hepatocytes (Figure 8A), uptake of 125I-LPS was significantly lower (by approximately 50%) in the hepatocytes, but not nonhepatocytes, of SR-BI–null mice compared with control mice (Figure 9A). Thirty minutes following injection, 125I-LPS uptake into the liver of SR-BI–null mice was significantly lower (by approximately 40%) than in control mice (Figure 9B). In contrast, uptake of 125I-LPS into the spleen and kidney was similar in the 2 animal groups (Figure 9B). In line with the reduced uptake of 125I-LPS into the liver of SR-BI–null mice, 125I-LPS remaining in plasma was greater in SR-BI–null mice. We conclude from these results that hepatic SR-BI expressed in hepatocytes plays a major physiologically significant role in clearance of LPS from plasma.

To assess whether the observed differences between control and SR-BI–null mice in response to LPS might be related to previously
show that SR-BI also plays a role in the clearance of plasma LPS. In addition to its role in glucocorticoid production, our results indicated that SR-BI–null mice failed to show any significant induction of corticosterone production in response to LPS. A lack of glucocorticoid supplementation at the relatively high levels reached in the SR-BI–null mice protects against endotoxemia in a manner unrelated to the adrenal insufficiency present in these mice. Since endotoxic shock is often associated with low levels of glucocorticoids, glucocorticoids have long been used in the management of sepsis and shown to efficiently limit uncontrollable inflammation (1). However, the concept of adrenal deficiency during sepsis is complex and is still in some doubt (28). Clinical trials, for example, have not shown survival benefit with the early use of high-dose corticosteroids in adrenally intact patients with severe sepsis (1). On the other hand, survival benefit was seen in a group of adrenally insufficient patients that did not respond to a corticotropin-stimulation test (28), and further clinical studies on the role of glucocorticoids in sepsis are therefore required.

During the acute phase of inflammatory response, the increased secretion of corticosterone depends on increased synthesis, a complex process involving a number of key regulatory steps. Our results show a normal increase in ACTH levels in SR-BI–null mice in response to LPS as well as a typical adrenal response to ACTH. Thus, LPS induced plasma levels of ACTH similar to those in control mice and also triggered typical ERK phosphorylation in the adrenals. Two other key regulatory proteins were shown to respond to stimulation of SR-BI–null mice similarly to control mice. StAR, which is required for cholesterol transport to the inner leaflet of mitochondria (29), and Cyp11A1, the side-chain cleavage enzyme responsible for pregnenolone formation (29), are both upregulated in adrenals in SR-BI–null mice as in control mice. Together, our data indicate normal ACTH release and consequent signaling in the adrenals in response to LPS and point to insufficient uptake of cholesterol required for glucocorticoid synthesis in SR-BI–null mice.

Corticosterone synthesis is dependent on an adequate supply of cholesterol to the adrenal cortex zona fasciculata cells. Cholesterol for steroid synthesis is derived largely from plasma lipoproteins in animals as well as humans (25, 40). Previous studies have shown that cholesterol can be taken up from LDL by SR-BI in the form of esterified and free cholesterol, although CEIs likely contribute to the majority of uptake (20, 41, 42). Cholesterol is also taken up from LDL by both the LDLR (23) and SR-BI (22, 43). However, HDL appears to be the more important source of cholesterol (24, 30). Consequently, apoA-I–null mice with low plasma HDL fail to accumulate CE in the adrenals and were shown to have a reduced glucocorticoid response to a swim stress test or ACTH challenge (30). Although SR-BI–null mice were found to have no gross phenotypic alterations, it was not clear whether these animals had an altered steroidogenic response (10). Our results show that during severe stress such as occurs in the response to LPS, SR-BI is absolutely required for enhanced corticosterone synthesis. Moreover, in addition to its role in the response to bacterial LPS, SR-BI was shown to be required for a normal glucocorticoid response to the conditions of a swim stress test. Thus, the critical role of SR-BI is not limited

**Figure 10**
Plasma from SR-BI+/+ and SR-BI–/– mice showed similar LPS neutralization activity. Plasma (1 µl) from SR-BI+/+ and SR-BI–/– mice (pooled from 4 mice in each group) was incubated with different amounts of LPS (10 ng, 25 ng, 50 ng, and 100 ng) at 37°C for 1 hour. Endotoxin activities relative to no-plasma controls at each LPS concentration (percentage of neutralization) were determined by using a commercially available LAL assay kit. Similar results were found in 3 independent experiments.

**Discussion**

The present study demonstrates that SR-BI–null mice exhibit an enhanced inflammatory response to LPS as shown by highly elevated levels of inflammatory cytokines compared with control mice and decreased survival when challenged with endotoxin. This occurs despite the elevated levels of HDL in these mice, which is normally protective against LPS-induced inflammation. Strikingly, SR-BI–null mice failed to show any significant induction of corticosterone production in response to LPS. A lack of glucocorticoid stimulation was also observed in these mice in response to exogenous ACTH, indicating that the glucocorticoid insufficiency is not due to impaired signaling in the hypothalamic-pituitary-adrenal axis upstream of the adrenals but rather is the result of primary adrenal deficiency due to an inadequate cholesterol supply. In addition to its role in glucocorticoid production, our results show that SR-BI also plays a role in the clearance of plasma LPS.

Our findings that corticosterone supplementation in SR-BI–null mice exerts a major protective effect against endotoxin-induced death provides strong support for a key protective role for glucocorticoids in endotoxin-induced death. Glucocorticoids act to efficiently limit the inflammatory response (39), and stimulation of glucocorticoid production by the adrenals has generally been considered a key component in host defense against severe inflammation and endotoxic shock (27). Our results further point to the strong possibility that SR-BI protects against endotoxemia in part through its role in glucocorticoid biosynthesis. However, since pharmacological doses of glucocorticoids have been shown to be protective even in the absence of adrenal insufficiency (28), it is possible that glucocorticoid supplementation at the relatively high levels reached in the SR-BI–null mice protects against endotoxemia in a manner unrelated to the adrenal insufficiency present in these mice. Since endotoxic shock is often associated with low levels of glucocorticoids, glucocorticoids have long been used in the management of sepsis and shown to efficiently limit uncontrollable inflammation (1). However, the concept of adrenal deficiency during sepsis is complex and is still in some doubt (28). Clinical trials, for example, have not shown survival benefit with the early use of high-dose corticosteroids in adrenally intact patients with severe sepsis (1). On the other hand, survival benefit was seen in a group of adrenally insufficient patients that did not respond to a corticotropin-stimulation test (28), and further clinical studies on the role of glucocorticoids in sepsis are therefore required.

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to the response of the foreign inflammatory agent LPS but is also important in the glucocorticoid response to physical and mental stress unrelated to bacterial infection.

The cholesterol supply in adrenals is potentially derived from 3 main sources, namely (a) CE from circulating HDL or LDL, (b) CE from CE droplets present in cells, and (c) endogenous cholesterol synthesis. While cholesterol synthesis may provide sufficient cholesterol for basal corticosterone synthesis, our results in SR-BI–null mice show that, under conditions of high demand, endogenous synthesis is unable to support increased corticosterone production. This occurs despite a marked induction of HMG-CoA reductase, the key regulatory enzyme in cholesterol biosynthesis. Similarly, despite its marked upregulation, the LDLR was also unable to support enhanced corticosterone production in these mice. This is in line with earlier findings that corticosterone production in LDLR-null mice is unimpaired (25). Our results clearly demonstrate that SR-BI–mediated selective CE uptake is essential for enhanced corticosterone production. The CE taken up by SR-BI serves as a source of cholesterol in CE droplets found in adrenocortical cells, as previously shown by the reduced cellular CE content in apoA-I–null and also SR-BI–null mice (10, 30). Such droplets are considered to be short-term stores of cholesterol for glucocorticoid synthesis, since they are rapidly depleted under ACTH stimulation (40). CE taken up by SR-BI may also be used more directly for glucocorticoid synthesis following its uptake and rapid hydrolysis by hormone-sensitive lipase (41). Hormone-sensitive lipase plays a key role in the hydrolysis of CE delivered to cells by SR-BI as well as CE stored in CE droplets (25). Although the SR-BI–null mice used in this study lack both SR-BI and SR-BII isoforms, SR-BI likely contributes to the vast majority of CE uptake, since SR-BII is expressed at low levels in the adrenals compared with SR-BI and is also less active in cholesterol uptake than SR-BI (17).

The critical role of SR-BI in glucocorticoid production suggests that downregulation of SR-BI–mediated HDL cholesterol delivery to the adrenals may be a mechanism contributing to glucocorticoid insufficiency, which is often observed during sepsis. During the inflammatory response to endotoxin, SR-BI has been shown to be downregulated in hepatocytes (44) and macrophages (45). Although the effect of LPS on SR-BI regulation in the adrenals is not known, downregulation of SR-BI would reduce cholesterol supply to the adrenal and may lead to decreased glucocorticoid production. Furthermore, the inflammatory response to endotoxin is typically characterized by a relatively rapid reduction in plasma levels of HDL cholesterol and apoA-I (46). Although the mechanisms responsible for this rapid decline in HDL remain uncertain, reduced HDL levels would also be expected to decrease SR-BI–mediated HDL cholesterol delivery to the adrenals. The combined effects of reduced HDL and SR-BI during inflammation may thus lead to significant reductions in glucocorticoid production.

Our finding that corticosterone supplementation did not completely normalize endotoxin-induced death in SR-BI–null mice, together with the fact that adrenalectomy did not completely abolish the difference in TNF-α response between SR-BI–null and control mice, indicates that SR-BI exerts other nonadrenal protective functions. SR-BI–null mice have abnormally large cholesterol-rich HDL particles due to defective HDL CE clearance (31). The apoA-I levels in normal and SR-BI–null mice are similar, suggesting that the number of HDL particles in the 2 mouse strains are also similar. Since lipoproteins such as LDL (5) and HDL (47) have strongly protective effects through their ability to neutralize LPS, we investigated whether an enhanced susceptibility of SR-BI–null mice to LPS might be due to an inability of the abnormal HDL in these mice to neutralize LPS. Our results, however, showed that the total LPS neutralizing activity of plasma from SR-BI–null and control mice was similar, thus showing effective binding and neutralization in both cases.

Earlier studies have shown that LPS, either in lipid-free form or when associated with HDL, is bound and internalized by SR-BI in HeLa cells and macrophages (15). On this basis, SR-BI might facilitate LPS clearance into hepatocytes, thereby decreasing the LPS burden. Our findings demonstrate that SR-BI indeed contributes significantly to LPS uptake into hepatocytes and to LPS clearance into the liver. In the case of nonhepatocytes, no significant difference was found in LPS association between control cells and SR-BI–null cells. Although earlier studies have pointed to the ability of Kupffer cells to take up and detoxify LPS, the mechanisms for this are ill-defined (8). However, our results suggest that the stimulation of inflammatory cytokines by LPS in Kupffer cells does not depend on the ability of SR-BI to bind or internalize LPS, as has been reported in the case of the THP-1, a human monocyte cell line (48). Thus, while SR-BI may serve other protective and antiinflammatory functions, these occur within the context of a powerful glucocorticoid response to LPS and proper clearance of LPS through the hepatocytes.

Sepsis is a complex process that can be divided into an early hyperinflammatory response stage that induces acute organ dysfunction and a later hypoinflammatory stage associated with immunosuppression (1). Treatment of sepsis is complicated, given the opposing natures of these 2 stages. Our findings suggest that SR-BI exerts protective functions throughout the process of sepsis, first by allowing a normal glucocorticoid response in the early hyperinflammatory stage and second, by limiting the LPS

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<td>Z-19521</td>
<td>5′-AGGCCTGTGGCCTGCCATAGG-3′ 5′-TGCGTGTCAGGGTCATCT-3′</td>
<td>1181-1252</td>
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<td>HMG-CoA reductase</td>
<td>NM_008255</td>
<td>5′-CTTTGGAATGCTTGGTATGG-3′ 5′-AGGCGAGGTCCACCATG-3′</td>
<td>578-653</td>
</tr>
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<td>TNF-α</td>
<td>NM_013693</td>
<td>5′-GGGAAAGTGCTTGGAGTATTG-3′ 5′-GTTAGAGATCAGACCTG-3′</td>
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<td>IL-6</td>
<td>NM_031168</td>
<td>5′-GCCATGATGCACTTGCAGG-3′ 5′-GTAAGCTATGGTACTCCAG-3′</td>
<td>266-400</td>
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<td>STAR</td>
<td>NM_011485</td>
<td>5′-TTGCGGATACTCAACACAC-3′ 5′-GAAACACCTTGCACAATCT-3′</td>
<td>312-414</td>
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<td>CYP11A1</td>
<td>NM_019779</td>
<td>5′-GACAGTCTCAGCCAGTACCC-3′ 5′-GCCCCAGCTCTGGAATTG-3′</td>
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<td>ABCA1</td>
<td>NM_013454</td>
<td>5′-AGGCAGAAGGAGATGTCAAG-3′ 5′-GATGACCTGCGTACACT-3′</td>
<td>2955-3056</td>
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*Numbers without parentheses refer to primer location on the gene. Numbers within parentheses refer to PCR product size.
burden during both stages by promoting LPS clearance. A recent study has reported that SR-BI protects against endotoxin-induced death by preventing NO-induced cytotoxicity (19). NO generation is induced during septic shock and contributes significantly to the symptoms of septic shock. Our results suggest that the glucocorticoid insufficiency seen in SR-BI–null mice might lead to enhanced endotoxic shock since glucocorticoids are known to inhibit NO production in a wide variety of cells (49). In the study by Li et al., however, NO levels in SR-BI–null mice were shown to be only moderately increased by LPS compared with control mice and the authors concluded that SR-BI prevents NO-induced cytotoxicity by mechanisms unrelated to NO synthesis (19).

Our data raise the possibility that the known protective role of SR-BI against atherosclerosis may be related to its antiinflammatory function. SR-BI–null mice and combined SR-BI/LDLR double-knockout mice on high-fat diets show accelerated atherosclerotic lesion development compared with control mice (50, 51). SR-BI/apoE–double-knockout mice not only develop atherosclerotic plaques but also have severe cardiac malfunction that includes spontaneous myocardial infarction and cardiac failure (52). Clearly, the lack of proper hepatic HDL cholesterol clearance will have significant effects in these SR-BI–null models. Nevertheless, our findings suggest that an enhanced inflammatory response may also contribute to atherogenesis in SR-BI–null mice. In SR-BI–null mice, for example, we showed that chemokines produced in macrophages and vascular cells, such as MCP-1 and RANTES, are markedly upregulated during inflammation. These chemokines have been reported to promote the initiation of plaque formation (53, 54). Studies using these models will need to take cognizance of possible effects of altered adrenal function. Furthermore, SR-BI may also play important roles in the clearance of endogenous proinflammatory reagents, including modified lipoproteins, such as oxidized LDL and other proinflammatory lipids, and pathogens. In contrast, mice lacking the class B scavenger receptor CD36, which reportedly can also clear LPS in vitro (18), did not show the exaggerated inflammatory response to LPS seen in SR-BI–null mice. In fact, although CD36–null macrophages showed a reduced cytokine response to LPS and E. coli challenge, CD36 deficiency in mice did not protect them from E. coli–induced lethality (55).

In summary, we demonstrate that SR-BI–mediated cholesterol delivery to the adrenals is essential for an effective antiinflammatory glucocorticoid response to LPS or stress, and SR-BI regulates LPS-induced inflammation and endotoxic shock. SR-BI serves a protective function in mediating LPS clearance from the plasma.

Methods

**Animals and reagents.** SR-BI–deficient mice (SR-BI null) were obtained from M. Krieger (Massachusetts Institute of Technology, Cambridge, Massachusetts, USA) (31). These mice lack both functional SR-BI and SR-BI. SR-BI homozygous (SR-BI+/−) mutant mice and control mice (both 1:1 mixed C57BL/6 × 129 backgrounds) were bred from 2 lines generated from a common mating pair of SR-BI heterozygous (SR-BI+/−) mice. Since female SR-BI−/− mice are infertile, SR-BI−/− mice were bred using SR-BI+/− male and SR-BI−/− female mice. For all animal experiments, 8- to 12-week-old male and female mice, weighing 20–25 g, were used. All animal experiments were approved by the Veterans Affairs Medical Center, Institutional Animal Care and Use Committee.

LPS (E. coli serotype 0111:B4; 1 × 10⁶ endotoxin units/ml) was obtained from Sigma-Aldrich. Corticosterone and ACTH ¹²⁵-I RIA assay kits were obtained from MP Biomedical. TNF-α and IL-6 ELISA kits were from R&D Systems, MCP-1 ELISA set was from BD Diagnostics, and the LAL assay kit was obtained from Cape Cod Inc.

E. coli (ATCC25922; ATCC) were cultured in Luria-Bertani broth and tryptic soy broth (ATCC) at 37°C using standard procedures and harvested at mid-log phase, which reached OD₅₆₀ 5.0 (2–10⁶ CFU/ml). Bacteria were washed twice, suspended in sterile saline, and injected i.p. into mice (2 × 10⁶ CFU E. coli/100 μl saline).

**Corticosterone and ACTH determination.** Plasma was isolated from blood obtained from SR-BI–null or control mice. Samples were assayed immediately to avoid degradation. Corticosterone levels and ACTH in the plasma were determined using specific radioimmunoassay kits (¹²⁵-I-RIA kit for ACTH and corticosterone; MP Biomedicals).

**Cytokine determination.** Serum was isolated from the blood of SR-BI–null and control mice and immediately frozen at −80°C. Cytokine levels were determined by multiplex biomarker assay using xMAP technology by Lincos Diagnostic Services Inc. Cytokine values were calculated using standard concentration curves determined for each cytokine on each 96-well plate used. Plasma TNF-α and IL-6 levels were also determined using ELISA kits from R&D systems; MCP-1 was determined using ELISA sets from BD Diagnostics.

**Q-PCR.** Total RNA was isolated from mouse liver and adrenals using the standard TRizol method (Invitrogen). RNA was further purified with DNase I (Roche) and RNeasy Mini Kit (QIAGEN). 2 μg of RNA was reverse transcribed into cDNA using a reverse-transcription system (Promega). After 4-fold dilution, 5 μl was used as a template for Q-PCR. Primers used in this study are listed in Table 2. Amplification was done for 40 cycles using Power SYBR Green PCR master Mix Kit (Applied Biosystems) and DNA Engine Optical 2 System (MJ Research Inc.). Both internal control (18S rRNA) and negative control (minus reverse transcriptase) were included. Values of each RNA sample were the average of duplicate assays normalized toward 18S rRNA (internal control) levels.

¹²⁵-I radioiodination and Alexa Fluor labeling of LPS. LPS was iodinated by the chloramine T method (56). In brief, 1 mg of LPS was dissolved in 0.05 M borate buffer (pH 8) and incubated with 50 mM pOH methylbenzimidate at 37°C for 18 hours. After extensive dialysis, the M-LPS was radioiodinated with Na¹²⁵I by the chloramine T method. After iodination, the unincorporated Na¹²⁵I was removed by extensive dialysis against sterile saline at 4°C. For fluorescent labeling of LPS, 1 mg of LPS was incubated with 1 vial of Alexa Fluor 488 dye (Invitrogen) in 0.1 M sodium borate buffer (pH = 10.5) for 3 hours at 37°C. After incubation, the mixture was dialyzed exhaustively against 4 liters of 150 mM NaCl with at least 6 changes. The bioactivity of labeled LPS was determined by the LAL assay kit. Near-identical bioactivity was observed among unlabeled LPS, ¹²⁵-I-LPS, and Alexa Fluor–LPS.

LPS neutralization assay. Lipoproteins were preincubated with various amounts of LPS at 37°C for 1 hour, after which LAL activity of LPS was quantitatively determined according to the manufacturer’s instructions. The analytical sensitivity of the kit is 0.005 EU/ml (0.5 pg of highly purified LPS/ml).

**Primary hepatocyte isolation and culture.** The isolation of primary hepatocytes and nonhepatocytes was performed as described earlier (57) with certain modifications. In brief, livers were minced and dissociated in collagenase containing medium at 37°C for 10–12 minutes. Cell suspensions were filtered through mesh (100 μm) and spun down. Hepatocytes were collected after a first spin (50 g for 2 minutes), while the nonhepatocytes were collected after a second spin (500 g for 10 minutes). Hepatocytes were washed twice in HBSS and suspended in enriched Williams’ E Medium (GIBCO; Invitrogen). Viable cells were counted and plated in 24-well mouse collagen IV-coated plates (Fisher).
LPS uptake into primary hepatocytes. Cell association assays were performed as described previously (13). In brief, primary hepatocytes were seeded into 24-well cell culture clusters at an initial density of 1 x 10^4 cells/cm². When cells reached confluency, they were washed with phosphate-buffered saline and incubated at 37 °C for 2 hours with 125I-LPS in DMEM medium containing 0.5% essentially fatty acid free albumin (BSA). After incubation, medium was removed and the cells were washed 4 times with cold buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing BSA (2 mg/ml) followed by 2 washes in the same buffer without BSA. The cells were then solubilized in 0.1 N NaOH for 1 hour at room temperature, and the protein and 125I content of the lysate were determined.

Adrenalectomy. SR-BI-null and control mice were adrenalectomized following standard surgical procedures (58). Following surgery, mice were placed in individual clean cages and allowed to recover for 1 week while maintained on normal chow diet and drinking water containing 0.9% sodium chloride. Sham-operated control mice underwent the same surgical procedure as adrenalectomized groups, except their adrenal glands were not excised. Experiments were performed 1 week after surgery. Sham-operated and test mice were injected i.p. with LPS and sacrificed 2 hours later.

Survival determination. SR-BI−/− mice, SR-BI+/− mice, and SR-BI+/+ mice were injected i.p. with 5 μg/g body weight of LPS. In some experiments, SR-BI−/− mice were given corticosterone (Sigma-Aldrich), initially dissolved in ethanol, in their drinking water (100 μg/ml) starting 8 hours prior to LPS injection. In these experiments, control groups were given an equivalent amount of vehicle only (0.1% ethanol) in their drinking water. Mice were allowed to eat and drink freely. Mice were under careful observation for behavior and survival during the following 3 days.

Swim stress test and ACTH treatment. For the stress test, female mice were placed in 5 °C water and forced to swim for 3 minutes (59). Following the swim, mice were rested at room temperature for 17 minutes prior to sacrifice. Blood was taken for corticosterone and ACTH determination. For ACTH treatment, mice were s.c. injected with the indicated amounts of ACTH (Questcor Pharmaceutical).


LPS clearance. SR-BI-null mice and control mice (n = 4) were injected with 0.125 μg/g body weight of 125I-I-LPS through tail veins. Mice were sacrificed after 30 minutes, and blood and tissue were collected for analysis. Plasma and tissue samples were weighed and counted in a γ counter (Packard, PerkinElmer Life Inc). Tissue uptake was determined as described (3).

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