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Endotoxin-Induced Cardiomyopathy and Systemic Inflammation in Mice Is Prevented by Aldose Reductase Inhibition

Kota V. Ramana, PhD; Monte S. Willis, MD, PhD; Michael D. White, BS; Jureta W. Horton, PhD; J. Michael DiMaio, MD; Deepak Srivastava, MD; Aruni Bhatnagar, PhD; Satish K. Srivastava, PhD

Background—Sepsis is a systemic inflammatory response syndrome characterized by excessive production of inflammatory cytokines and cardiovascular collapse. Postreceptor signaling events that lead to stress responses and cytokine production are sensitive to redox changes and products of lipid peroxidation.

Methods and Results—We tested the hypothesis that inflammatory signaling and cytokine generation during sepsis depend on the activity of the enzyme aldose reductase, which catalyzes the reduction of lipid peroxidation–derived aldehydes and their glutathione conjugates. The results of the present study show that pharmacological inhibition of aldose reductase by sorbinil or knockdown of the enzyme by small interfering RNA prevents the activation of nuclear factor- κ B and the release of tumor necrosis factor- α from lipopolysaccharide-stimulated RAW264.7 or H9c2 cells. Increases in serum and cardiac cytokines in response to lipopolysaccharide challenge were suppressed by inhibition of aldose reductase. Treatment with sorbinil blunted the activation of protein kinase C, c-Jun NH₂-terminal kinase, and p38, as well as phosphorylation of interleukin receptor–associated kinase, I κ B- α , I κ B kinase complex- α/β , and phospholipase- γ 1 and - β 1. These changes were associated with decreased myocardial nuclear factor- κ B and activating protein-1 activity, prostaglandin E2 production, induction of cyclooxygenase 2, and inducible nitric oxide synthase. Sorbinil treatment also induced functional recovery in myocardial fractional shortening in vivo and preserved contractile function of isolated perfused hearts. Inhibition of aldose reductase increased survival in mice injected with lethal doses of lipopolysaccharide.

Conclusions—The present demonstration that aldose reductase mediates endotoxin-induced inflammation and cardiomyopathy suggests that inhibition of this enzyme may be useful to attenuate maladaptive host responses and to treat acute cardiovascular dysfunction associated with endotoxic shock. (*Circulation*. 2006;114:1838-1846.)

Key Words: cardiomyopathy ■ infection ■ inflammation ■ inhibitors ■ signal transduction

Lipopolysaccharide (LPS) is a component of the outer envelope of all Gram-negative bacterial cell walls.¹ During infection, LPS is released in the circulation, where it stimulates the pattern-recognizing toll-like receptor (TLR4)-MD2-CD14 receptor complex on circulating cells.^{2,3} Cells that lack constitutive TLR4 receptors, such as vascular endothelial and smooth muscle cells, respond to LPS by interacting with soluble CD14.^{4,5} Ligation of LPS with CD14 activates these receptors. This increases the transcription of inflammatory and immune-response genes via mechanisms that depend on dissociation of the I κ B–nuclear factor (NF)- κ B complex and translocation of NF- κ B to the nucleus, where it activates cytokine gene promoters.^{2,6} Stimulation of the NF- κ B pathway leads to increased transcription of several

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proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin (IL)-1, as well as prostaglandins and nitric oxide (NO). Acting in an autocrine and paracrine manner, these and other cytokines amplify host responses to invading pathogens.^{7,8} Although activation of the innate immune response during host-pathogen interaction is initially adaptive, the inability to regulate immune responses,⁹ which causes immunoparalysis,⁴ leads to sepsis. Clinically, this results in vasodilatory shock, circulatory collapse, and often fatal myocardial depression.¹⁰ Although sepsis is associated with a high (30% to 50%) risk of mortality, microbial diagnosis is made in only half the cases.⁴ Similar symptoms

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of systemic inflammatory syndrome also develop in patients with severe heart failure who are treated with mechanical assist devices and those who have undergone prolonged cardiopulmonary bypass.¹⁰ Currently, few therapeutic options are available, and the mechanisms underlying the pathogenic development of this syndrome remain incompletely understood.^{4,9,10}

Uncontrolled cytokine production mediates several of the immunopathological features of LPS-induced shock.¹¹ The cytokines released early, usually within 30 to 90 minutes of infection,⁸ stimulate a broad array of secondary responses that include changes in coagulation, vascular reactivity, cell adhesion, and myocardial contractility. In addition, some cytokines, particularly TNF- α and IL-1 β , stimulate gene transcription via the NF- κ B pathway, which leads to further increases in NO and cytokine production.^{4,8,10} Nevertheless, direct interruption of cytokine production has not been efficacious in the clinical management of sepsis.^{9,11} More effective therapeutic interventions are required, preferably to interrupt regenerative cycles of cytokine synthesis and the uncontrolled progression of inflammation.

Regenerative production of cytokines is perpetuated by repeated cycles of NF- κ B activation.^{4,8,11} NF- κ B, initially activated by ligation of the TLR4 receptor complex, increases the transcription of TNF- α and IL- β genes. These cytokines in turn cause further activation of NF- κ B through paracrine and autocrine effects that lead to amplification of cytokine synthesis.⁸ Therefore, inhibition of NF- κ B represents 1 approach for interrupting regenerative cytokine production and unresolved inflammation. Our previous studies showed that NF- κ B activation could be prevented by inhibiting the enzyme aldose reductase (AR), which regulates postreceptor events that lead to stimulation and nuclear translocation of NF- κ B.^{12,13} Thus, inhibition of AR may be 1 method of preventing NF- κ B activation during sepsis.

AR is a member of the aldo-keto reductase superfamily classified as aldo-keto reductase 1B2.¹⁴ It is a cytosolic protein that catalyzes nicotinamide adenine dinucleotide phosphate-dependent reduction of a wide range of aldehydes.¹⁵ AR displays high catalytic efficiency with aldehyde products of lipid peroxidation^{16,17} and their conjugates with glutathione.^{16,18,19} The enzyme also catalyzes the reduction of glucose to sorbitol, and an increase in AR-mediated metabolism of glucose has been linked to the development of secondary diabetic complications.^{15,20} Our recent studies show that inhibition of AR in vascular smooth muscle cells prevents diacylglycerol synthesis, thereby attenuating the activation of several protein kinase C (PKC) isoforms and downstream signaling events that lead to the activation of NF- κ B.²¹ Inhibition of these signaling events prevents high glucose- and TNF- α -induced vascular smooth muscle growth^{12,22} and TNF- α -induced vascular endothelial cell apoptosis.¹³ Inhibitors of AR also prevent NF- κ B activation in vivo in arterial lesions, and hence, they decrease neointimal hyperplasia in balloon-injured arteries.¹² On the basis of these observations, we tested the hypothesis that inhibition of AR would prevent systemic inflammation and cardiac dysfunction during LPS-induced shock. Our results showing marked antiinflammatory effects of the inhibition of AR

suggest that inhibition of AR could represent one approach for keeping renegade inflammatory responses in check.

Methods

Cell Culture and Animals

The C57BL/6 mice (weight \approx 25 g) were obtained from the Jackson Laboratory (Bar Harbor, Me) and housed in pathogen-free conditions with free access to food and water at the institutional animal care facility. RAW264.7 macrophage cell line and H9C2 rat cardiac myoblasts were obtained from American Type Culture Collection (Manassas, Va) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and in accordance with the "Guideline of the Animal Care and Use Committee" of the University of Texas Medical Branch at Galveston and Southwestern Medical Center at Dallas, Tex.

Antisense Ablation of AR

Antisense ablation of AR was performed as described previously.¹² Small interfering RNAs (siRNA) were designed to target the coding sequence (AATCGGTGTCTCCAACCTCAA) of mouse AR (GenBank accession No. BC085310). A scrambled oligonucleotide AR siRNA (AAAATCTCCCTAAATCATACA) was used as a control. RAW264.7 cells were grown in DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C and 5% CO₂ and seeded on 6-well plates. When the cells reached 60% to 70% confluence (in 24 hours), the medium was replaced with fresh DMEM without serum, and the cells were incubated with the siRNA to a final concentration of 100 nmol/L and with the RNAiFect transfection reagent (Qiagen, Valencia, Calif) as per the supplier's instructions. After incubation for 15 minutes at 25°C, the medium was aspirated and replaced with fresh DMEM containing 10% serum, added dropwise to the cells. The cells were cultured for 48 hours at 37°C (5% CO₂), and changes in AR expression were determined by Western blot analysis with anti-AR antibodies and by measuring AR activity in the total cell lysates.¹²

Biochemical Measurements

The mice were preinjected with sorbinil (25 mg/kg body weight IP) or carrier, and after 24 hours, LPS (4 mg/kg body weight) was injected with or without sorbinil. The RAW264.7 cells were preincubated with 10 μ g/mL sorbinil for 24 hours followed by incubation with 1 μ g/mL LPS. At various time intervals, the animals were killed, and blood and heart tissues were collected. Cytokine (TNF- α , IL-6, IL-12, and interferon [IFN]- γ) and chemokine (monocyte chemoattractant protein-1) levels were measured in the serum and heart homogenates with BD Biosciences mouse inflammation cytometric bead array kits (BD Biosciences, San Jose, Calif). Cyclooxygenase-2, prostaglandin E₂, and nitrate/nitrite levels in heart homogenates were determined with commercially available assay kits obtained from Cayman Chemical (Ann Arbor, Mich). The nuclear extracts were prepared, and electrophoretic mobility gel shift assay was performed as described previously.¹² The NF- κ B activity was determined by electrophoretic mobility gel shift assay or with the colorimetric nonradioactive NF- κ B p65 Transcription factor assay kit (Chemicon, Temecula, Calif). Activating protein-1 (AP-1) activity was determined by electrophoretic mobility gel shift assay and PKC activation with the Promega SignaTECT total PKC assay system (Promega, Madison, Wis), as described previously.¹²

Western Blot Analysis

Equal amounts of cell extracts or heart homogenates were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electroblotted on nitrocellulose membranes, and probed with phospho- and nonphospho-specific antibodies against phospholipase C (PLC)- β 1 PLC- γ 1, I κ B kinase complex (IKK), interleukin receptor-associated kinase (IRAK)-1, I κ B- α , p38-mitogen-activated protein (MAP) kinase, c-Jun NH₂-terminal kinase (JNK), or extracellu-

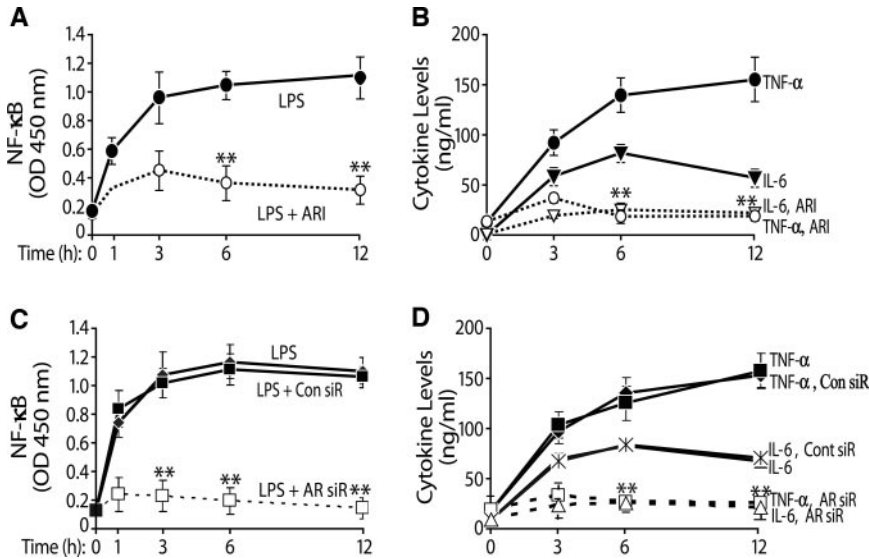


Figure 1. Inhibition of AR prevents LPS-induced NF- κ B activation and cytokine production in macrophages. RAW264.7 cells were growth-arrested in DMEM containing 0.1% serum without or with 10 μ M sorbinil for 24 hours and then challenged with LPS (1 μ g/mL). The cells were transfected with AR siRNA (AR siR) or scrambled (cont siR) oligonucleotides and challenged with LPS (1 μ g/mL) as described in Methods. Cells were harvested at indicated times and used for measuring NF- κ B activity (A, C) and TNF- α and IL-6 levels (B, D). Values are mean \pm SEM (n=4). ** P <0.001 vs LPS-treated cells.

lar signal-regulated kinase 1/2. Antibody binding was detected by enhanced Pico chemiluminescence (Pierce, Rockford, Ill). Immunopositive bands were quantified with Kodak Image station 2000R loaded with Kodak 1-dimensional image-analysis software (Kodak, Rochester, NY).

Cardiac Dysfunction Assessment by Echocardiography

Echocardiography was performed on 5 groups of mice: (1) control mice (no treatment); (2) mice treated with sorbinil for 5 days, starting 3 days before echocardiography; (3) mice challenged with LPS (4 mg/kg IP in 200 μ L of phosphate-buffered saline); (4) mice treated with sorbinil (as in group 2) and LPS challenge (as in group 3) on day 3; and (5) mice treated with sorbinil 2 hours after LPS challenge. Pretreatment with sorbinil (25 mg \cdot kg $^{-1}$ \cdot d $^{-1}$) was followed by LPS challenge. Serial echocardiograms to assess cardiac function were performed with M-mode measurements as described previously²³ (online-only Data Supplement). M-mode measurement data represent the average of 9 selected cardiac cycles from at least 2 separate scans. End diastole was defined as the maximal left ventricular diastolic dimension, and end systole was defined as the peak of posterior wall motion. Fractional shortening percentage (FS%), a surrogate of systolic function, was calculated from left ventricular dimensions as follows

$$(1) \quad FS\% = \frac{LVED - LVES}{LVED} \times 100,$$

in which LVED indicates left ventricular end-diastolic diameter and LVES is left ventricular end-systolic diameter.

Ex Vivo Cardiac Function Determination by Langendorff

Mouse heart function was determined by the Langendorff assay procedure as described previously.²⁴ Procedural details are described in the online Data Supplement.

Statistical Analysis

Cardiac function determined by the Langendorff preparation (including stabilization data) is expressed as mean \pm SEM, and separate analyses were performed for each left ventricular pressure, $+dP/dt_{max}$, and $-dP/dt_{max}$, as a function of treatment group and perfusate Ca $^{2+}$ concentration (or constant coronary flow for stabilization measurements) with a repeated-measures ANOVA. A multiple-comparison procedure with the Bonferroni correction was used to determine the significance of differences between groups. Cardiac function determined by M-mode echocardiography is expressed as FS% \pm SE and was analyzed by

1-way repeated-measures ANOVA. Additional comparisons were performed with the Tukey test to determine significant differences between specific groups. Statistical significance for all analyses was defined as P <0.01. Survival rates were compared by the Kaplan-Meier method and analyzed by log-rank test. All statistical analyses were performed with SigmaStat 2.03 (SPSS, Chicago, Ill) and Microsoft Office Excel (Microsoft, Seattle, Wash).

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Inhibition of AR Prevents LPS-Induced Activation of NF- κ B and Cytokine Release in Macrophages

We first examined how inhibition of AR would affect NF- κ B activation and cytokine production in LPS-stimulated macrophages. Treatment of RAW264.7 macrophages with LPS led to a 6-fold increase in NF- κ B activity within 6 hours of stimulation, and it remained elevated at this level for 12 hours. Treatment with the AR inhibitor sorbinil suppressed the initial increase in NF- κ B activity, and after 12 hours, the increase in NF- κ B activity was not statistically different from the values obtained before LPS stimulation. Sorbinil treatment also blunted LPS-induced increases in TNF- α and IL-6 (Figure 1B).

Although sorbinil is a relatively specific AR inhibitor, its nonspecific effects cannot be rigorously excluded. Therefore, we examined the effects of ablating the AR transcripts using siRNA. As shown in Figure I in the online Data Supplement, 48 hours after transfection, the level of AR protein in RAW264.7 macrophages transfected with AR siRNA was <5% of the level in untransfected cells or cells transfected with control siRNA. Consistent with the pharmacological data, transfection with AR siRNA, but not with scrambled oligonucleotides, attenuated LPS-induced NF- κ B activation and cytokine production (Figures 1C and 1D). Pharmacological inhibition of AR by sorbinil or tolrestat or siRNA knockdown of AR mRNA also significantly prevented LPS-induced NF- κ B and AP-1 activation and synthesis of TNF- α and IL-6 in the cardiomyocyte-derived H9c2 cell line (Data Supplement, Figure II). These findings suggest that inhibition

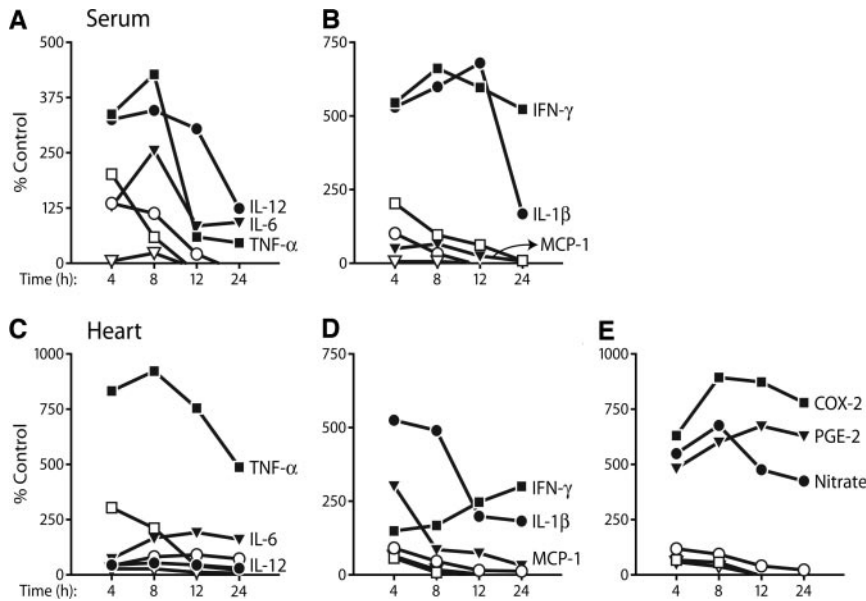


Figure 2. Inhibition of AR prevents cytokine generation in LPS-treated mice. C57BL/6 mice (n=6 per group) were injected with sorbinil or vehicle every day for 3 days and then challenged with LPS (4 mg/kg body weight). At the indicated times, levels of TNF- α , IL-6, IL-12, IFN- γ , IL-1 β , and monocyte chemoattractant protein (MCP)-1 were determined in the serum (A, B) and in the heart (C, D). Additionally, levels of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE-2), and nitrates were measured in the heart (E). Filled symbols represent values from mice injected with LPS; empty symbols represent values from mice treated with LPS and sorbinil. Statistically analyzed group data are listed in supplementary Table I.

of AR suppresses NF- κ B-mediated gene transcription in LPS-treated cells of macrophage or cardiac origin.

Inhibition of AR Prevents LPS-Induced Inflammation In Vivo

To determine whether inhibition of AR would also prevent systemic inflammation in vivo, we examined the effects of AR inhibition on NF- κ B signaling pathways and myocardial dysfunction in a mouse model of LPS-induced shock. After treatment with sorbinil or vehicle alone, mice were injected intraperitoneally with a sublethal dose of LPS (4 mg/kg body weight). Changes in inflammatory cytokines and chemokines were measured in the serum and the heart. In LPS-treated mice, the levels of TNF- α , IL-6, IL-12, IL-1 β , and IFN- γ increased in the serum by 3- to 6-fold after 8 hours of LPS exposure (Figure 2A and 2B). The levels of these cytokines began declining by 12 hours but remained elevated for 24 hours after treatment. In sorbinil-treated mice, the increase in serum cytokine levels at different time intervals was significantly less than that with LPS alone (Figure 2A and 2B). Sorbinil treatment also prevented inflammatory markers in the heart. Within 4 hours of LPS treatment, levels of TNF- α , IL-1 β , and monocyte chemoattractant protein-1 in the heart were markedly elevated (Figure 2C and 2D). No significant increase in IL-12 was observed, and the increase in IL-6 was small. The levels of IFN- γ were elevated by 2.5-fold within 4 hours and continued to increase for the next 24 hours. In sorbinil-treated mice, cardiac cytokine production was largely abrogated, and only small increases in TNF- α , IFN- γ , and monocyte chemoattractant protein-1 were noted, which were significantly smaller than in mice treated with LPS alone. Treatment with LPS also increased the myocardial content of cyclooxygenase-2 protein, prostaglandin E2, and nitrate (Figure 2E), which were markedly blunted in sorbinil-treated mice. The efficacy of sorbinil in preventing an LPS-induced increase in serum cytokines was similar in mice that were treated with sorbinil either 3 days before LPS injection or 2 hours after LPS injection (Data Supplement, Table I). Fur-

thermore, comparable changes in TNF- α , IL-6, and IFN- γ were observed in mice treated with 2 other structurally unrelated AR inhibitors, tolrestat and zopolrestat (Data Supplement, Figure III). Collectively, these data suggest that inhibition of AR interrupts and suppresses LPS-induced increases in plasma and cardiac cytokines, even in a therapeutic setting.

To determine whether AR inhibition could rescue cardiac dysfunction associated with systemic inflammation, we performed serial echocardiography. After 4 hours of LPS injection, the FS% decreased from 50% to 25% (Figure 3A). A similar decrease in FS% was initially observed in mice treated with sorbinil and LPS; however, 8 hours after LPS treatment, the sorbinil-treated mice showed significant recovery compared with mice treated with LPS alone. After 48 hours, the FS% in sorbinil-treated mice was >40%, whereas it remained near 25% in those treated with LPS alone (Figure 3A). FS% was not affected in unchallenged controls or in mice treated with sorbinil in the absence of LPS. Similar results were observed in mice given sorbinil 2 hours after LPS challenge (data not shown). The general activity level of LPS-exposed mice was consistent with the echocardiographic findings: Despite LPS injection, the sorbinil-treated mice exhibited normal grooming and other activities within 24 hours, whereas the mice treated with LPS alone remained inactive and huddled close to one another.

To further assess the effect of AR inhibition on cardiac function, we perfused spontaneously beating isolated mouse hearts in the Langendorff mode with the AR inhibitor and challenged them with LPS. Hearts perfused with LPS alone showed diminished contractility, which was more pronounced at high calcium levels and high coronary flow rates, which indicates that LPS treatment depresses the positive inotropic responses to calcium and coronary flow. The attenuation of calcium and coronary flow-dependent changes in LPS-treated hearts was reflected in both positive and negative dp/dt_{max} , the velocity of contraction and relaxation, respectively (Figure 3B and 3C). The time to maximal

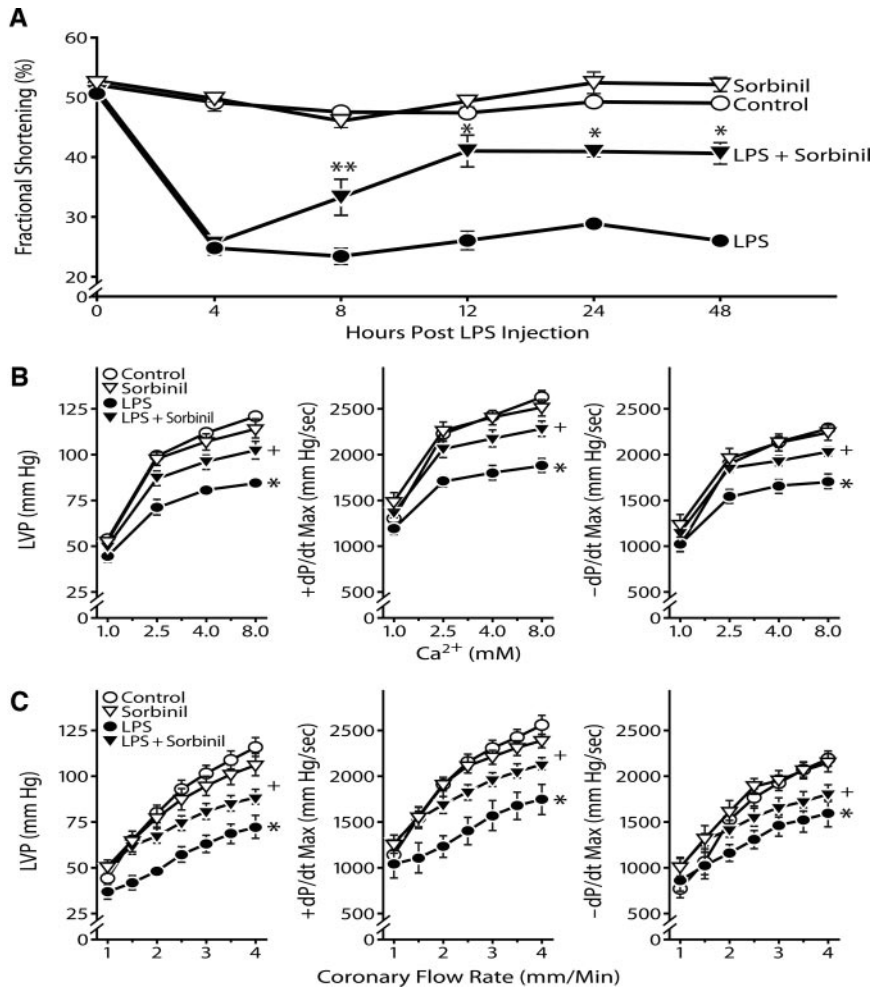


Figure 3. Inhibition of AR protects against LPS-induced cardiac dysfunction. A, Adult male C57BL/6 mice (n=6 per group) were injected with sorbinil or vehicle for 3 days and then challenged with LPS (4 mg/kg body weight). FS% was determined by M-mode echocardiography 0 to 48 hours after LPS injection. Values are mean±SEM (n=6). **P<0.01 and *P<0.001 vs LPS. B and C, Cardiac function in isolated mouse hearts perfused in the Langendorff mode at various times after LPS challenge as a function of increasing Ca²⁺ concentration (B) or coronary flow rate (C). Values are mean±SEM of 6 independent experiments. *P<0.001 vs control and +P<0.01 vs LPS.

±dP/dt, coronary perfusion pressure, coronary vascular resistance, and heart rate were unaffected. Treatment with sorbinil significantly prevented the loss of inotropy and enhanced the sensitivity of the heart to calcium and coronary flow (Figure 3B and 3C). These findings demonstrate that inhibition of AR activity attenuates LPS-induced systolic and diastolic dysfunctions.

Inhibition of AR Suppresses LPS Lethality

To determine whether in addition to preventing sublethal effects of LPS, inhibition of AR also affects LPS lethality, we examined whether treatment with sorbinil would affect LPS-induced death. The effects of sorbinil were studied with 2 different protocols. In protocol 1, the mice were pretreated with sorbinil for 3 days before LPS treatment, whereas in protocol 2, the mice were treated with sorbinil 2 hours after injection of LPS. In mice that were pretreated with carrier alone, the lethal dose of LPS for 50% of the population was ≈14 mg/kg body weight over 48 hours. Both protocols of sorbinil treatment, however, resulted in significantly higher survival. As shown in Figure 4, 16 mg of LPS per kilogram of body weight resulted in only 25% survival, whereas in the sorbinil-pretreated group (protocol 1), none of the mice died. In the group in which sorbinil was injected 2 hours after LPS (protocol 2), only 4 mice died (66.6% survival). Even at a

dose of 24 mg/kg body weight, at which 100% of the control mice died, 60% of mice survived in the LPS plus sorbinil-pretreatment group, and 40% of mice survived in the sorbinil-posttreatment group. Additional statistical analysis of the survival rate at each dose of LPS compared by the Kaplan-Meier method and analyzed by log-rank test is given as supplementary Figure IV and supplementary Tables II and III. Taken together, these data support the notion that inhibition of AR allows mice to survive a potentially lethal LPS challenge.

Inhibition of AR Extinguishes LPS Signaling

We next asked whether the cardioprotective effects of sorbinil might reflect suppression of the inflammatory signaling cascade. In untreated mice, LPS increased cardiac NF-κB activation 16-fold and AP-1 activation 5-fold within 2 hours (Figure 5A and 5B; supplementary Figure V). The level of these transcription factors remained elevated even 24 hours after LPS challenge. Inhibition of AR severely blunted these responses. In sorbinil-treated mice, LPS-induced activation of NF-κB and AP-1 was decreased by ≈70% at 2 hours compared with mice treated with LPS alone. In the presence of sorbinil, the levels of these transcription factors were close to baseline values by 24 hours. Similar results were observed in mice treated with sorbinil 2 hours after LPS exposure (Data

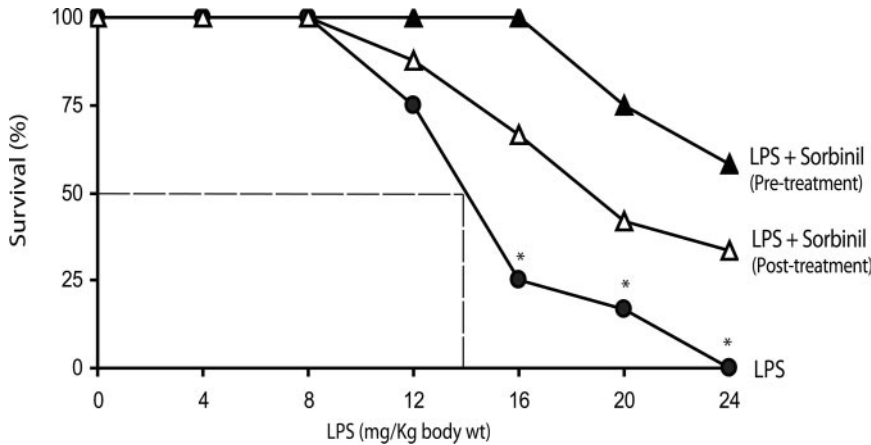


Figure 4. Protective effect of AR inhibition on LPS lethality. Adult male C57BL/6 mice (n=12 mice per group) were injected with sorbinil or vehicle 24 hours before (pretreatment) or 2 hours after (posttreatment) challenge with the indicated concentrations of LPS; survival percentage after 48 hours is plotted. Doses at which 50% lethality occurred (LD₅₀) are indicated. *P<0.001 vs without sorbinil treatment. Statistically analyzed group data are listed in supplementary Tables II and III.

Supplement, Figure VI). Furthermore, subsequent to LPS injection, cardiac PKC activity was increased by 5-fold in 1 hour, and it remained ~3-fold elevated for the next 24 hours (Figure 5C). In mice pretreated with sorbinil, the increase in PKC activity was significantly less. In addition, the LPS-induced increase in phosphorylation of PLC-β1 and -γ1 was significantly attenuated by AR inhibition (Figure 6). Similarly, cardiac inducible NO synthase levels were increased by ~3-fold 8 hours after LPS injection and remained elevated for 24 hours (Figure 5D). In sorbinil-treated mice, however, inducible NO synthase levels increased only slightly and returned to baseline levels in 24 hours. Thus, inhibition of AR appears to attenuate NF-κB activation and protects against cardiovascular collapse in the setting of overwhelming sepsis.

To further assess the extent to which inhibition of AR interrupts signaling events downstream of TLR4 activation, we examined changes in IRAK and MAP kinases in hearts of mice treated with LPS. Previous studies show that TLR4 stimulation leads to phosphorylation of IRAK and the activation of p38 and JNK via a TAK1 (transforming growth factor-β-activated kinase)-dependent pathway that also activates NF-κB signaling.⁶ In agreement with these observations, we found that LPS stimulated the phosphorylation of IRAK, p38, and JNK (Figure 6). IRAK phosphorylation was

increased 3-fold within 15 minutes of stimulation with LPS. Maximal MAP kinase activation (~8-fold) was observed at 30 minutes, and the activation was sustained up to 120 minutes for p38 and up to 90 minutes for JNK. In contrast, extracellular signal-regulated kinase was only mildly activated (~2-fold) after 90 to 120 minutes of stimulation. Treatment with sorbinil suppressed LPS-induced increases in IRAK, p38, and JNK, whereas extracellular signal-regulated kinase was only marginally affected (Figure 6). In addition, inhibition of AR also significantly prevented LPS-induced activation of IKK and IκB-α phosphorylation. Inhibition of IKK was particularly profound, and a weak phospho-IKK signal was observed only transiently at 90 minutes. Collectively, these data suggest that inhibition of AR does not directly interfere with NF-κB but interrupts signaling events upstream of IKK activation.

Discussion

Results of the present study show for the first time that inhibition of AR, an enzyme involved in glucose and aldehyde metabolism, prevents the cytokine production and cardiac dysfunction associated with the systemic inflammatory response syndrome in LPS-treated mice. The present studies suggest that the salutary effects of AR inhibition may

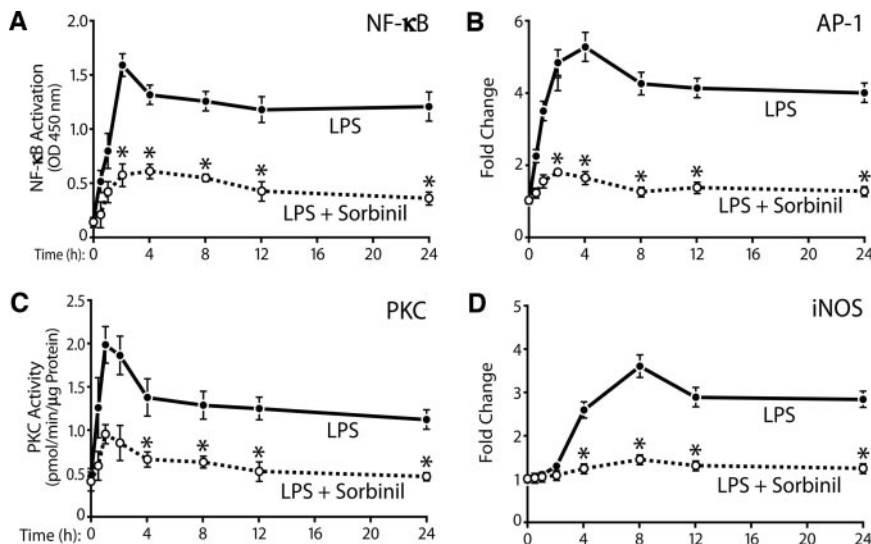


Figure 5. Effect of AR inhibition on LPS-triggered signaling in the heart. Adult male C57BL/6 mice were injected with sorbinil or vehicle for 3 days and then challenged with LPS (4 mg/kg body weight). At the indicated times, the activities of NF-κB (A), AP-1 (B), PKC (C), and inducible NO synthase protein (D) were measured in the hearts of mice treated with LPS alone (solid lines) or sorbinil and LPS (broken lines). Values represent mean ± SEM (n=6 mice per group). *P<0.001 vs the group treated with LPS alone.

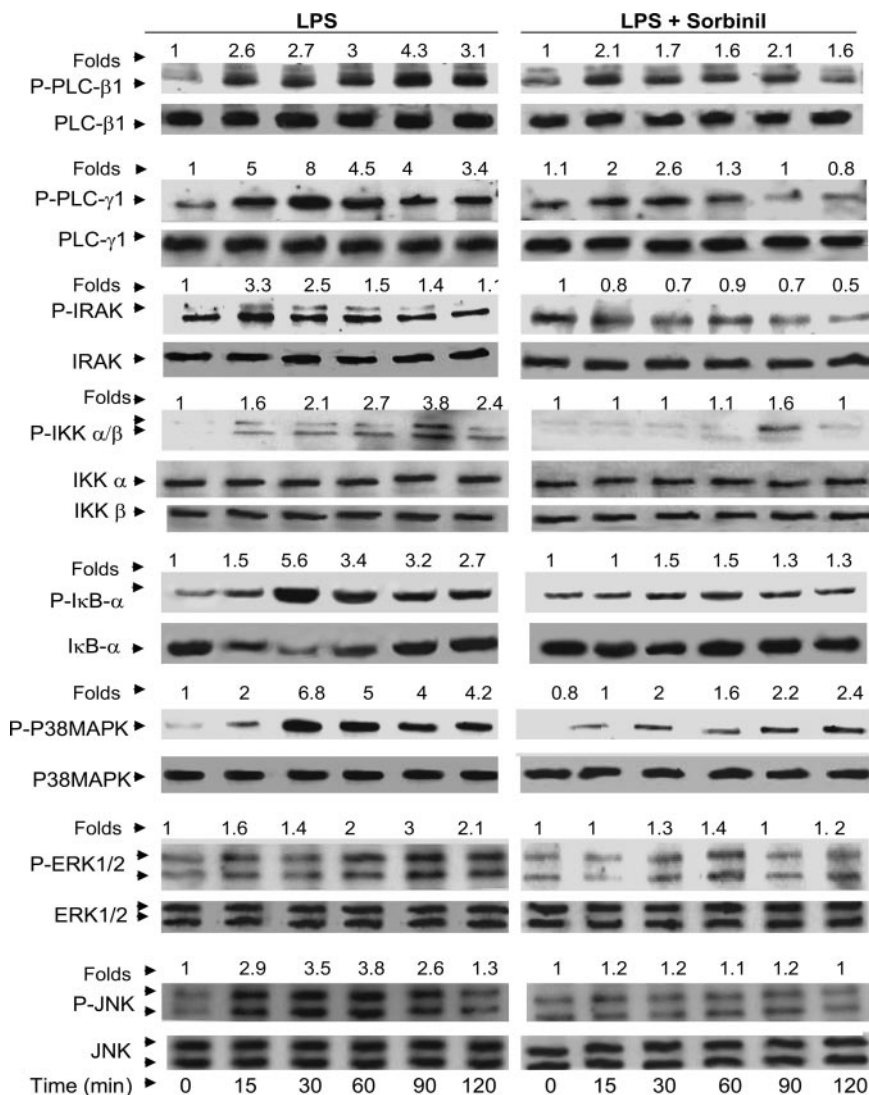


Figure 6. Inhibition of AR prevents LPS-induced activation of protein kinases in the heart. Adult male C57BL/6 mice ($n=6$ per group) were injected with sorbinil or vehicle for 3 days and then challenged with LPS (4 mg/kg body weight). At the indicated times (0 to 120 minutes), the levels of individual nonphosphoproteins and phosphoproteins were measured in heart homogenates by Western blot analysis with antibodies against respective proteins. The fold change in the phosphorylated protein, normalized with total protein, determined by densitometry analysis is indicated at the top of each panel.

be related to the inhibition of inflammatory signaling mediated by transcription factors (NF- κ B and AP-1) and stress-activated MAP kinases (JNK and p38). These results raise the possibility that inhibition of AR may be beneficial in treating sepsis and other clinical conditions associated with maladaptive inflammation.

A variety of clinical conditions are associated with a dysregulation of inflammatory responses. Although the most common of these is sepsis, high concentrations of cytokines are also generated by trauma, ischemia-reperfusion, acute rejection, antigen-specific immune responses, and several acute inflammatory states, such as acute hepatitis and pancreatitis.¹⁰ Given that tissue dysfunction in these conditions has been linked to excessive inflammation, it appears likely that as in sepsis, increased cytokine production in these conditions could also be mediated by AR. Hence, excessive cytokine production under these conditions could be prevented by the inhibition of AR. A general role of AR in mediating inflammation and cytokine generation is consistent with recent observations showing that inhibition of AR prevents PKC and NF- κ B activation by a variety of stimuli such as TNF- α , fibroblast growth factor-2, platelet-derived

growth factor-AB, angiotensin II, and high glucose^{12,13,21,22} and high glucose-induced phosphatidylinositol-3 kinase²⁵ and Janus kinase 2,²⁶ which suggests that AR regulates stress response and the activation of NF- κ B and other PKC-sensitive transcription factors.

Activation of NF- κ B starts with the assembly of a multi-protein complex comprising IKK α /IKK β proteins held together with a scaffolding protein, IKK γ (NEMO). This complex phosphorylates I κ B- α , which then dissociates from NF- κ B and becomes ubiquitinated and degraded.⁷ The present finding that LPS-stimulated phosphorylation of IKK α / β and I κ B- α is prevented by sorbinil (Figure 6) suggests that inhibition of AR interferes with events that occur before the formation of the IKK α / β signalosome. In LPS signaling, the IKK α / β complex is assembled via a TAK1-dependent pathway that also activates JNK and p38.⁶ The observation that phosphorylation of JNK and the p38 kinases was severely attenuated by sorbinil further indicates that the signals that precede TAK1 activation, such as IRAK activation, are prevented by sorbinil treatment and that inhibition of AR does not directly interfere with NF- κ B, stress-activated protein kinase, or their downstream effectors.

LPS-triggered signaling events further upstream of IKK activation are mediated by the activation of PKC. Macrophage PKC activity is increased by LPS stimulation, and PKC inhibitors prevent LPS-induced NF- κ B activation and the release of NO, TNF- α , and IL-1 β .^{27,28} In agreement with a central role of PKC, we found a marked PKC activation within 2 hours of stimulation with LPS (Figure 5C). Sorbinil treatment prevented LPS-induced activation of PKC, which supports our previous observation that inhibition of AR prevents PKC activation.^{12,21,22} The present results, however, do not rule out the possibility that inhibition of AR could prevent inflammatory signaling by preventing PKC-independent signaling pathways. The present *in vivo* experiments show that treatment with AR inhibitors prevented cytokine production even when the drug was administered 2 hours after LPS challenge, although in the *in vitro* experiments, PKC and NF- κ B activations were early events (<2 hours). Hence, either sustained activation of PKC and NF- κ B is required for *in vivo* effects or other non-PKC- or NF- κ B-dependent mechanisms may be responsible for the effects of AR inhibitors. Nevertheless, even though the mechanism by which AR facilitates PKC signaling remains unclear, we propose that inhibition of AR prevents events that lead to the activation of phosphatidylcholine-PLC, which is activated by LPS.²⁹ We have recently reported that inhibition of AR prevents phosphatidylinositol-PLC-dependent synthesis of diacylglycerol.²¹ A similar mechanism may be responsible for the effects of AR inhibition on LPS-induced PLC- β 1, PLC- γ 1, PKC, and NF- κ B activation. Alternatively, inhibition of AR could affect signaling owing to products of lipid peroxidation or their glutathione conjugates. In this regard, it is interesting that the oxidized phospholipids such as 1-palmitoyl, 2-oxovaleryl phosphocholine (POVPC), which is a substrate of AR,¹⁷ inhibit NF- κ B activation and increase survival in mice injected with lethal doses of LPS.³⁰ Thus, inhibition of AR could potentially prevent inflammation by allowing oxidized phospholipids to accumulate. However, oxidized phospholipids bind LPS and thereby prevent its binding to CD14 and LPS-binding protein and its presentation to TLR4.³⁰ These lipids do not inhibit TNF- α -induced NF- κ B activation,³⁰ which is sensitive to AR inhibition.¹² Hence, changes in phospholipid metabolism are unlikely to account fully for the antiinflammatory effects of AR inhibition, although the possibility that multiple mechanisms contribute to the *in vivo* efficacy of AR inhibition in preventing sepsis cannot be ruled out.

Regardless of the specific mechanisms involved, the present data showing prevention of NF- κ B and AP-1 activation by sorbinil in myocardial tissue of LPS-treated mice suggest that this is an *in vivo* phenomenon and not restricted to isolated cells in culture. This view is further reinforced by the observation that sorbinil prevented the rise in myocardial cytokines (Figure 2) and the activation of cyclooxygenase-2 (Figure 2E) and inducible NO synthase (Figure 5D) activity in the heart, an observation that inhibition of AR prevents not only the activity of inflammatory transcription factors but also the products of the genes they activate.

Our observation that inhibition of AR decreases cytokine release and promotes recovery of cardiac function suggests

that this enzyme is a critical modifier of LPS toxicity. Because excessive cytokine production is a significant problem in sepsis, treatment with AR inhibitors may be useful in attenuating both cytokine generation and their ability to induce tissue dysfunction. That inhibition of AR could affect tissue responses directly is suggested by our previous observation that inhibition of AR blocks TNF- α -induced NF- κ B activation in endothelial cells and smooth muscle cells.¹³ The present data showing that inhibition of AR preserves cardiac function in an isolated perfused heart preparation further underscores the significance of AR in mediating oxidative stress signals. Hence, inhibition of AR could diminish not only the extent to which cytokines are synthesized but also the intensity with which tissues respond to cytokine challenge.

It is significant that treatment with sorbinil was able to prevent inflammation even when the therapy was initiated after LPS challenge. One of the reasons the clinical efficacy of anticytokine production drugs cannot be tested is that cytokines are released in the early stages of infections, ie, patients present too late for the therapy to be effective.⁴ Hence, we propose that the inhibition of metabolic pathways, which simultaneously decreases both cytokine production and toxicity, is likely to be a more tractable approach because it could treat secondary tissue inflammation even if it is too late to prevent early systemic release of cytokines. Moreover, because mortality is not primarily due to the infection but to the secondary tissue dysfunction and multiorgan failure,^{4,10} it appears likely that sepsis deaths could be prevented by AR inhibitors. In summary, the present results provide the first line of evidence for an unanticipated role of an aldehyde-metabolizing enzyme in mediating acute inflammatory responses and provide a new concept that inhibition of AR might be useful in preventing the clinical sequelae of sepsis or acute hemorrhagic or cardiogenic shock.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Bacterial infections can result in overwhelming septic shock, which equates to a mortality rate of 30% despite antibiotic therapy. Antibiotic-induced lysis of bacteria results in a concentrated release of cell-wall lipopolysaccharide (LPS) that induces reactive oxygen species, which stimulate a nuclear factor (NF)- κ B-dependent activation of cytokines and chemokines. This uncontrolled host response is deleterious and results in cardiovascular collapse from vascular and myocardial dysfunction. Here, we show that the enzyme aldose reductase (AR) mediates bacterial endotoxin LPS-induced signaling cascade involving phosphorylation of phospholipase C, protein kinase C, and I κ B, which ultimately results in nuclear translocation of NF- κ B. Inhibition of AR in macrophages disrupted this signaling cascade and blunted the cytokine response to LPS. Correspondingly, AR inhibition prevented LPS-induced NF- κ B activation and cytokine release in vivo and prevented the cardiovascular collapse and mortality typically associated with bacterial sepsis. The present study describes a novel role for AR in blunting a subset of deleterious NF- κ B-dependent inflammatory processes; hence, the inhibition of AR may be a useful approach to attenuate maladaptive host responses and treat acute cardiovascular collapse due to endotoxic shock.

SUPPLEMENTAL METHODS

Materials: Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), penicillin/streptomycin solution, trypsin, and fetal bovine serum (FBS) were purchased from Invitrogen. Sorbinil was a gift from Pfizer. Normal or phospho-specific antibodies against PLC-b1, PLC γ 1, p38-MAPK, JNK, IKK, ERK1/2, I κ B- α were obtained from Cell Signaling Inc. Mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase antibodies were obtained from Research Diagnostics Inc. Cyclooxygenase (COX) activity assay and prostaglandin E2 (PGE2) assay and nitrate/nitrite assay kits were obtained from Cayman chemical company. Calorimetric non-radioactive NF- κ B p65 Transcription Factor Assay kit was obtained from Chemicon Int. Consensus oligonucleotides for NF- κ B and AP-1 were obtained from Promega. Lipopolysaccharide (*E.coli*) and the reagents used in the electrophoretic mobility shift assay (EMSA) and Western blot analysis were obtained from Sigma. All other reagents used were of analytical grade.

Cell culture and animals: The C57BL/6 mice (25-30 g) obtained from Jackson's laboratories were housed in pathogen-free conditions with free access to food and water at the institutional animal care facility. The RAW264.7 murine macrophages and H9C2 rat cardiac myoblasts were obtained from ATCC were grown in DMEM containing 10 % FBS.

Cytokine Measurements: The mice were pre-injected with sorbinil (25 mg/Kg body wt, i.p.) or carrier. After 24 h, the mice were injected with LPS (4 mg/kg body wt) without or with sorbinil. The RAW264.7 cells were pre-incubated with 10 μ M of sorbinil for 24 h, after which 1 μ g/ml LPS was added to the medium. The animals were killed at different times and blood and heart tissues were collected. The cytokine (TNF- α , IL-6, IL-12, IFN- γ) and chemokine (MCP-1) levels were measured in the heart homogenates and serum by using BD Biosciences Mouse Inflammation Cytometric bead array kits according to manufacturer's instructions.

Measurements of PGE-2 and nitrate/nitrite levels: The PGE2 and nitrate/nitrite levels in heart homogenates were determined using commercially available assay kits from Cayman Chemical.

Measurements of NF- κ B activity: Cytosolic as well as nuclear extracts were prepared and EMSA was performed as described before (1). The NF- κ B activity was determined by 1) electrophoretic mobility gel shift assay (EMSA) or by using the calorimetric non-radioactive NF- κ B p65 Transcription Factor Assay kit (Chemicon) as per manufacturer's instructions. Briefly, a double stranded biotinylated oligonucleotide containing the consensus sequence for NF- κ B binding (5'-GGGACTTTCC-3') was mixed with nuclear extract assay buffer provided. After incubation, the mixture (probe+extract+buffer) was transferred to the streptavidin-coated ELISA plate. The ELISA kit was developed, stopped and

read at 450 nm on an ELISA plate reader. For each experiment, samples were measured in triplicate to calculate statistical significance.

Measurements of AP-1 activity: The nuclear extract were prepared from the cultured cells and from the heart tissue and EMSA for AP-1 was performed as described before (1).

Measurements of PKC Activity: Membrane-bound and total PKC activities were measured by using the Promega Signa TECT PKC assay system according to the manufacturer's instructions and as described earlier (1). Briefly, aliquots of the reaction mixture (25 mM Tris-HCl pH 7.5, 1.6 mg/ml phosphatidylserine, 0.16 mg/ml diacylglycerol, and 50 mM MgCl₂) were mixed with [γ -³²P] ATP (3,000 Ci/mmol, 10 μ Ci/ μ l) and incubated at 30°C for 10 min. The extent of phosphorylation was detected by measuring the radioactivity retained on the paper.

RNA interference ablation of AR in RAW264.7 cells: The ablation of AR mRNA was essentially carried out as described earlier (2). Briefly, RAW264.7 cells were incubated with serum-free medium containing the AR-siRNA (AATCGGTGTCTCCAACCTTCAA) or scrambled siRNA (AAAATCTCCCTAAATCATACA; control) to a final concentration of 100 nM and the RNAiFectTM transfection reagent (Qiagen). After 15 min of incubation at 25 °C, the medium was aspirated and replaced with fresh DMEM containing 10 % serum. The cells were cultured for 48 h at 37 °C (5% CO₂), and AR expression was determined by measuring AR protein by Western blot using anti-AR antibodies and by measuring AR activity in the total cell lysates (2).

RNA interference ablation of AR in H9C2 cells: The ablation of AR mRNA was essentially carried out as described earlier (2). Briefly, rat cardiac myocytes were incubated with serum-free medium containing the AR-siRNA or scrambled siRNA to a final concentration of 500 nM and the RNAiFect™ transfection reagent (Qiagen). After 15 min of incubation at 25 °C, the medium was aspirated and replaced with fresh DMEM containing 10 % serum. The cells were cultured for 48 h at 37 °C (5% CO₂), and AR expression was determined by measuring AR protein by Western blot using anti-AR antibodies and by measuring AR activity in the total cell lysates (2).

Western blot analysis: Cell extracts or heart homogenates containing equal amounts of protein were separated on 12% SDS-PAGE and subsequently electro-blotted on nitrocellulose membranes and probed with phospho- and non-phospho-specific antibodies of PLCβ1, PLCγ1, IKK, IRAK-1, IκB-α, P38-MAPK, JNK and ERK1/2. The antibody binding was detected by enhanced pico chemiluminescence (Pierce). Immunopositive bands were quantified using Kodak Image station 2000R loaded with Kodak 1D image analysis software.

Determination of Cardiac Dysfunction by Echocardiography

Echocardiography was performed on five groups of mice: 1) control mice (no treatment), 2) mice treated with sorbinil for 5 days, starting 3 days prior to echocardiography, 3) mice challenged with LPS (4 mg/kg, i.p. in 200 μl PBS), 4) mice treated with sorbinil (as in group 2) and LPS (as in group 3) on day 3. Pre-treatment with sorbinil (25 mg/kg/day) was continued after LPS challenge. Serial echocardiograms to assess cardiac function were performed using M-mode

measurements. Mice were lightly anesthetized with 5% isoflurane with 2.5 L/m O₂ for 20 seconds (until unconsciousness) followed by 2% isoflurane and O₂ for an average of 12-15 minutes. Hair was removed from the thorax and upper abdomen using Nair® hair remover and gauze after sitting for 3 minutes. Echocardiographic measurements were obtained on anesthetized mice approximately 5-8 minutes after induction as previously described (3). Echocardiography was performed using an Acuson Sequoia™ Model C256 (Siemens Medical Solutions, USA, Inc., Mountain View, CA) with a frame rate of 300-500 frames/second in a random and blinded manner. A 15 MHz linear transducer (15L8, Siemens Medical Solutions, USA, Inc.) was placed on the left hemithorax interfaced with a layer of ultrasound transmission gel (Aquasonic 100, Parker Laboratories; Fairfield, NJ). The two dimensional parasternal short-axis imaging plane guided LV M-mode tracings close to the papillary muscle level. Depth was set at a minimum of 2 cm with a sweep speed of 200 m/second.

M-mode measurements:

Data represent the average of nine selected cardiac cycles from at least two separate scans. End diastole was defined as the maximal LV diastolic dimension, and end systole was defined as the peak of posterior wall motion. Fractional shortening % (FS%), a surrogate of systolic function, was calculated from LV dimensions as follows: $FS (\%) = \frac{LVED - LVES}{LVED} \times 100$.

Isolated perfused heart preparation:

Cardiac function was determined in isolated perfused heart preparations as described before (4). Briefly, 200 units of heparin sulfate were injected

intraperitoneally, and the mice were killed 20 min later. The heart was removed immediately and placed on ice in Krebs-Henseleit buffer [2 mM NaHCO₃, 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, and 11.1 mM glucose (pH 7.4), which was prepared fresh with demineralized, deionized water and bubbled with 95% O₂-5% CO₂ (PO₂ 590 mmHg, PCO₂ 38 mmHg)]. The aorta was cannulated with polyethylene (PE)-50 tubing, and the heart was perfused in a retrograde manner through the aortic root with prefiltered, oxygenated Krebs-Henseleit buffer at a constant flow rate of 1.5 ml/min (temperature 37°C, 100 ml recirculating volume). The heart was placed in a water-jacketed chamber to maintain constant temperature and humidity. PE-60 intramedic polyethylene tubing, connected to a Statham pressure transducer, was inserted into the LV to measure LV pressure. Temperature was monitored by a 27-gauge thermistor needle inserted into the LV muscle. After instrumentation, the hearts were allowed to stabilize for 10 min. Hearts that failed to achieve a stable pressure or developed persistent arrhythmias during this time were excluded from the study [only 1 heart (sham) failed to achieve stability during the present study and was excluded]. After stabilization, LV pressure and its first derivative (dP/dt), heart rate, and coronary perfusion were measured simultaneously with a multichannel Grass 7D polygraph (Grass Instruments, Quincy, MA). Ventricular performance as a function of Ca²⁺ concentration was determined for all hearts by plotting peak systolic LV pressure and maximum positive and negative dP/dt ($\pm dP/dt_{\max}$) values against incremental increases in perfusate Ca²⁺ concentration flow rate.

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FIGURE LEGENDS:

Fig. S1: Ablation of AR by RNA interference in Raw264.7 cells. The serum-starved Raw264.7 cells were transfected with double-stranded aldose reductase-specific siRNA to a final concentration of 100 nmol and cultured for 48 h at 37°C. The cells that were incubated with the transfection reagent only or with nonspecific RNA (control siRNA) were used as control. A and B, Western blot analysis of Raw264.7 cell extracts were developed using anti-aldose reductase or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies, respectively.

Fig. S2: Inhibition or ablation of AR prevents LPS-induced NF- κ B, AP-1 activation and cytokine production in cardiac myocytes (H9C2 cells). H9C2 cells were growth-arrested in Dulbecco's modified Eagle's medium containing 0.1% serum without or with 10 μ M sorbinil for 24 h and then challenged with LPS (1 μ g/ml). The cells were transfected with AR siRNA (AR siR) or scrambled (cont siR) oligonucleotides. The cells

were challenged with LPS (1 $\mu\text{g}/\text{ml}$), harvested at indicated times and used for measuring (A, B) NF- κ B activity, (C) TNF- α and (D) IL-6 levels as described in the text. Values are given as mean \pm SEM ($n = 4$) $**P < 0.001$ versus LPS-treated cells. Insert to panel C shows Western blot analysis for AR protein in untransfected and siRNA transfected cells. (UN, untransfected; TR, transfection reagent; S, scrambled siRNA; AR, AR siRNA)

Fig. S3: Inhibition of aldose reductase by three structurally distinct pharmacological inhibitors prevents cytokine generation in LPS-treated mice. C57BL/6 mice ($n = 4$ per group) were injected with indicated AR inhibitors or vehicle every day for 3 days and then challenged with LPS (4 mg/kg body wt). At the indicated times, levels of TNF- α , IL-6 and IFN- γ were determined in the serum (**left panel**) and in the heart (**right panel**) by using specific ELISA kits as described in the methods. Bars represent mean \pm SEM ($n = 4$), * $P < 0.001$ compared with LPS- treated versus LPS+ AR inhibitor- treated. # $P < 0.001$ compared LPS-treated versus vehicle-treated control.

Fig. S4: Survival rates of LPS-injected mice in the absence and presence of AR inhibitors compared by Kaplan-Mier method: Adult male C57BL/6 mice ($n = 8$ mice per group) were injected with sorbinil or vehicle for 24 h before (pre-treatment) or 2 h after (post-treatment) challenging with indicated concentrations of LPS. Survival distribution function at various time points at a given LPS dose of A) 12 B) 16 C) 20 and D) 24 mg/Kg body wt were compared by Kaplan-Mier method and plotted using Sigmaplot8.0 software and data shown in Table -S2 was analyzed by a log-rank test.

Table-S3 shows survival data at 48 h after LPS treatment were analyzed by both chi-square and the Fisher exact test of contingency tables.

Fig. S5: Representative EMSA for NF- κ B and AP-1 activation in heart (for Fig 5A and 5B): Adult, male C57BL/6 mice were injected with sorbinil or vehicle for 3 days and challenged with LPS (4 mg/kg body wt). At the indicated times, the activity of NF- κ B and AP-1 were measured in the nuclear extracts prepared from the mouse hearts by EMSA as described in the methods.

Fig. S6: Effect of AR inhibition on LPS –induced NF- κ B and AP1 in mice heart: The C57BL/6 (~25 g) mice were injected LPS (4 mg/kg body wt. i.p). After 2 h mice were injected with sorbinil (25 mg/kg body wt. i.p) or carrier. At the indicated time intervals, the following parameters were measured A, NF- κ B activation by Chemicon Int, transcription factor assay kit; B, AP1 activation by EMSA; AP1 activity was expressed as fold change as compared to control and was determined after densitometric analysis of gels. The figure is the representative of one of the three independent analyses (n=3).

TABLE S1 A:

HEART	Control	Sorbiniil	LPS				LPS + Sorbiniil (pre-injected)			
			<i>Time h</i>	<i>0</i>	<i>4</i>	<i>8</i>	<i>12</i>	<i>24</i>	<i>4</i>	<i>8</i>
TNF- α (pg/mg protein)	62.56 \pm 26.79	71.36 \pm 21.26	583.8 \pm 87.52**	635.5 \pm 163.52**	535.68 \pm 102.36**	368.5 \pm 45.6**	288.62 \pm 56.36##	223.60 \pm 49.63##	101.23 \pm 54.26##	88.5 \pm 32.4##
IL-6	21.78 \pm 4.67	19.82 \pm 3.8	36.67 \pm 6.81*	58.32 \pm 12.02**	63.85 \pm 13.74**	56.59 \pm 17.89**	27.89 \pm 6.98#	35.76 \pm 10.18#	38.45 \pm 7.65##	33.54 \pm 6.23##
IL-12	75.38 \pm 16.82	67.89 \pm 12.68	109.24 \pm 23.45*	115.45 \pm 12.89*	108.49 \pm 21.38*	99.78 \pm 15.52*	85.79 \pm 14.35#	82.24 \pm 18.79#	79.25 \pm 12.87#	73.62 \pm 10.69#
IL-1 β	12.35 \pm 2.34	12.58 \pm 4.69	76.87 \pm 18.89**	72.36 \pm 16.45**	36.69 \pm 10.23**	34.89 \pm 3.34**	23.45 \pm 11.39##	17.85 \pm 7.57##	14.24 \pm 6.90##	13.56 \pm 3.96##
INF- γ	39.45 \pm 4.66	43.39 \pm 12.23	98.34 \pm 12.78**	104.67 \pm 32.56**	134.87 \pm 23.56**	158.87 \pm 37.67**	67.45 \pm 23.67#	45.89 \pm 11.92##	41.02 \pm 12.67##	36.12 \pm 8.79##
MCP-1	223.57 \pm 45.79	259.76 \pm 87.89	898.68 \pm 124.67**	412.12 \pm 79.98**	387.70 \pm 124.98**	289.98 \pm 69.98 ^{NS}	412.98 \pm 109.89##	301.68 \pm 87.09##	258.89 \pm 57.78##	249.02 \pm 58.97 ^{NS}
COX-2 (U/mg protein)	79.09 \pm 21.34	82.60 \pm 14.67	569.78 \pm 79.56**	789.89 \pm 134.81**	768.12 \pm 127.89**	696.45 \pm 109.78**	137.89 \pm 45.92##	128.08 \pm 26.78##	79.67 \pm 12.87##	70.09 \pm 15.78##
PGE-2 (ug/ mg protein)	8.09 \pm 1.78	7.79 \pm 2.87	46.90 \pm 10.89**	56.78 \pm 12.36**	62.69 \pm 21.03**	58.68 \pm 10.90**	12.56 \pm 2.90##	10.86 \pm 2.78##	7.76 \pm 1.0##	6.89 \pm 2.27##
Nitrate (uM)	3.23 \pm 0.89	3.56 \pm 1.09	20.89 \pm 5.78**	25.12 \pm 8.96**	18.59 \pm 10.90**	16.89 \pm 8.97**	7.89 \pm 2.8##	6.82 \pm 2.78##	4.98 \pm 2.6##	4.29 \pm 1.18##
SERUM	Control	Sorbiniil	LPS				LPS + Sorbiniil (pre-injected)			
<i>Time (h)</i>	<i>0</i>	<i>0</i>	<i>4</i>	<i>8</i>	<i>12</i>	<i>24</i>	<i>4</i>	<i>8</i>	<i>12</i>	<i>24</i>
TNF- α	34.90 \pm 6.19	27.32 \pm 9.6	152.5 \pm 24.98**	185.25 \pm \pm 33.31**	55.6 \pm 10.6**	50.51 \pm 5.26*	82.23 \pm 16.36##	43.20 \pm 9.35##	21.43 \pm 4.63##	18.35 \pm 4.14##
IL-6	19.18 \pm 3.97	21.24 \pm 5.3	43.47 \pm 10.81**	68.54 \pm 12.02**	35.53 \pm 9.47*	36.19 \pm 14.91*	22.29 \pm 4.38##	25.73 \pm 6.18##	18.52 \pm 2.65##	15.54 \pm 5.33##
IL-12	56.35 \pm 9.26	57.59 \pm 10.84	239.45 \pm 43.45**	252.4 \pm 22.93**	228.42 \pm 41.48**	125.7 \pm 22.52**	135.79 \pm 24.52##	122.24 \pm 12.79##	69.22 \pm 11.71##	46.35 \pm 8.96##
IL-1 β	9.52 \pm 2.43	11.52 \pm 3.23	59.81 \pm 8.92**	66.22 \pm 12.25**	74.18 \pm 12.23**	26.44 \pm 9.14**	23.25 \pm 7.39##	15.25 \pm 2.52##	10.42 \pm 2.20##	8.12 \pm 2.62##
INF- γ	29.42 \pm 2.43	28.92 \pm 4.52	188.24 \pm 23.8**	224.27 \pm 24.56**	204.87 \pm 43.55**	182.82 \pm 32.37**	87.25 \pm 33.72##	55.92 \pm 9.91##	47.12 \pm 9.72##	32.12 \pm 4.73##
MCP-1	67.72 \pm 15.29	59.26 \pm 8.92	98.28 \pm 11.67*	112.22 \pm 12.98**	82.20 \pm 14.82*	69.38 \pm 9.98 ^{NS}	62.8 \pm 19.91##	61.62 \pm 11.92##	56.89 \pm 7.82##	59.92 \pm 8.79#

Table S1 A: Effect of AR inhibitor injected prior to LPS- challenge on mice serum and heart cytokines. The C57BL/6 (25 g) mice were pre-injected with sorbiniil (25 mg/Kg body wt, i.p/day) for three days followed by LPS injection (4 mg/kg body wt). At indicated time intervals, the levels TNF- α , IL-6, IL-12, INF- γ , IL-1 β and MCP-1 were measured in the serum and heart homogenates by using BD biosciences Mouse Inflammation Cytometric bead array kits according to manufacturer's instructions. The levels of PGE2, COX-2 and Nitrate were measured by using specific ELISA kits as described in methods. The data represents Mean \pm SEM (n=6). **P<0.001, *0.01, NS Non-significant as compared to control and ## P<0.001, #P<0.01 as compared to LPS injected mice.

TABLE S1 B:

HEART	Control		Sorbiniil		LPS		LPS + Sorbiniil (2 h after LPS injection)	
	Time h	0	0	12	24	12	24	
TNF- α (pg/mg protein)		62.56 \pm 26.79	71.36 \pm 21.26	535.68 \pm 102.36 ^{**}	368.5 \pm 45.6 ^{**}	161.63 \pm 24.16	158.23 \pm 12.41	
IL-6		21.78 \pm 4.67	19.82 \pm 3.8	63.85 \pm 13.74 ^{**}	56.59 \pm 17.89 ^{**}	42.12 \pm 6.35	35.45 \pm 7.32	
IL-12		75.38 \pm 16.82	67.89 \pm 12.68	108.49 \pm 21.38 [*]	99.78 \pm 15.52 [*]	77.5 \pm 9.72	67.22 \pm 11.92	
IL-1 β		12.35 \pm 2.34	12.58 \pm 4.69	36.69 \pm 10.23 ^{**}	34.89 \pm 3.34 ^{**}	18.43 \pm 3.90	15.62 \pm 4.56	
INF- γ		39.45 \pm 4.66	43.39 \pm 12.23	134.87 \pm 23.56 ^{**}	158.87 \pm 37.67 ^{**}	73.12 \pm 22.62	56.72 \pm 10.21	
MCP-1		223.57 \pm 45.79	259.76 \pm 87.89	387.70 \pm 124.98 [*]	289.98 \pm 69.98 ^{NS}	288.12 \pm 37.23	221.12 \pm 28.02	
COX-2 (U/mg protein)		79.09 \pm 21.34	82.60 \pm 14.67	768.12 \pm 27.89 ^{**}	696.45 \pm 109.78 ^{**}	212.27 \pm 32.87	92.23 \pm 9.23	
PGE-2 (ug/ mg protein)		8.09 \pm 1.78	7.79 \pm 2.87	62.69 \pm 21.03 ^{**}	58.68 \pm 10.90 ^{**}	15.5 \pm 2.12	10.19 \pm 1.37	
Nitrate (uM)		3.23 \pm 0.89	3.56 \pm 1.09	18.59 \pm 10.90 ^{**}	16.89 \pm 8.97 ^{**}	7.78 \pm 4.2	5.59 \pm 1.3	

SERUM	Control		Sorbiniil		LPS		LPS + Sorbiniil (2 h after LPS injection)	
	Time (h)	0	0	12	24	12	24	
TNF- α		34.90 \pm 6.19	27.32 \pm 9.6	55.6 \pm 10.6 [*]	50.51 \pm 5.26 [*]	32.31 \pm 6.21 [#]	21.25 \pm 3.43 [#]	
IL-6		19.18 \pm 3.97	21.24 \pm 5.3	35.53 \pm 9.47 [*]	36.19 \pm 14.91 [*]	23.22 \pm 6.35 [#]	18.24 \pm 2.45 ^{##}	
IL-12		56.35 \pm 9.26	57.59 \pm 10.84	228.42 \pm 41.48 ^{**}	125.7 \pm 22.52 ^{**}	106.32 \pm 21.67 ^{###}	82.25 \pm 11.62 [#]	
IL-1 β		9.52 \pm 2.43	11.52 \pm 3.23	74.18 \pm 12.23 ^{**}	26.44 \pm 9.14 [*]	32.32 \pm 4.5 ^{###}	14.22 \pm 5.12 ^{##}	
INF- γ		29.42 \pm 2.43	28.92 \pm 4.52	204.87 \pm 43.55 ^{**}	182.82 \pm 32.4 ^{**}	157.2 \pm 12.24 [#]	74.22 \pm 6.33 ^{##}	
MCP-1		67.72 \pm 15.29	59.26 \pm 8.92	82.20 \pm 14.82 [*]	69.38 \pm 9.98 ^{NS}	62.12 \pm 8.43 [#]	55.26 \pm 5.69 ^{NS}	

Table S1 B: Effect of AR inhibitor injected after LPS- challenge on mice serum and heart cytokines. The C57BL/6 (25 g) mice were injected LPS (4 mg/kg body wt. i.p) subsequently after 2 h mice were injected without or with sorbiniil (25 mg/kg body wt. i.p). At indicated time intervals, the levels TNF- α , IL-6, IL-12, INF- γ , IL-1 β and MCP-1 were measured in the serum and heart homogenates by using BD biosciences Mouse Inflammation Cytometric bead array kits according to manufacturer's instructions. The levels of PGE2, COX-2 and Nitrate were measured by using specific ELISA kits as described in methods. The data represents Mean \pm SEM (n=6). The data represents Mean \pm SEM (n=6). **P<0.001, *0.01, NS Non-significant as compared to control and ## P<0.001, #P<0.01 as compared to LPS injected mice.

Table-S2: Analysis of survival rate by log-rank test.

LPS dose (mg/kg body wt)	Comparison	Log Rank P value
12	Gp1 vs Gp2	0.0625
	Gp1 vs Gp3	0.2595
	Gp2 vs Gp3	0.3173
16	Gp1 vs Gp2	0.0833
	Gp1 vs Gp3	0.0291
	Gp2 vs Gp3	0.5832
20	Gp1 vs Gp2	0.0042
	Gp1 vs Gp3	0.02
	Gp2 vs Gp3	0.5243
24	Gp1 vs Gp2	0.003
	Gp1 vs Gp3	0.0822
	Gp2 vs Gp3	0.2283

Treatments: Gp1: LPS alone; Gp2: LPS + ARI pretreated; Gp3: LPS + ARI post-treated.
Number mice in each group =12.

Table-S3: Comparison of survival rate for pre or post -ARI treated mouse at 48h, by LPS concentration.

A

LPS (mg/kg body wt)	LPS alone (Gp1) (% Live mice)	LPS+ARI-1 (Gp2) (% Live mice)	<i>P</i> value*
0	100	100	-
4	100	100	-
8	100	100	-
12	75	100	0.46
16	25	100	0.0003
20	16.6	75	0.0123
24	0	58.3	0.0046

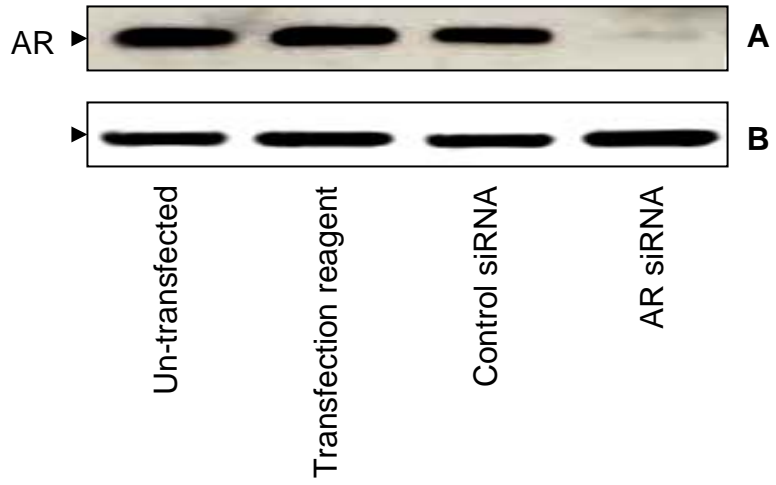
* Fisher's Exact Test; (number of animals in each group =12)

B

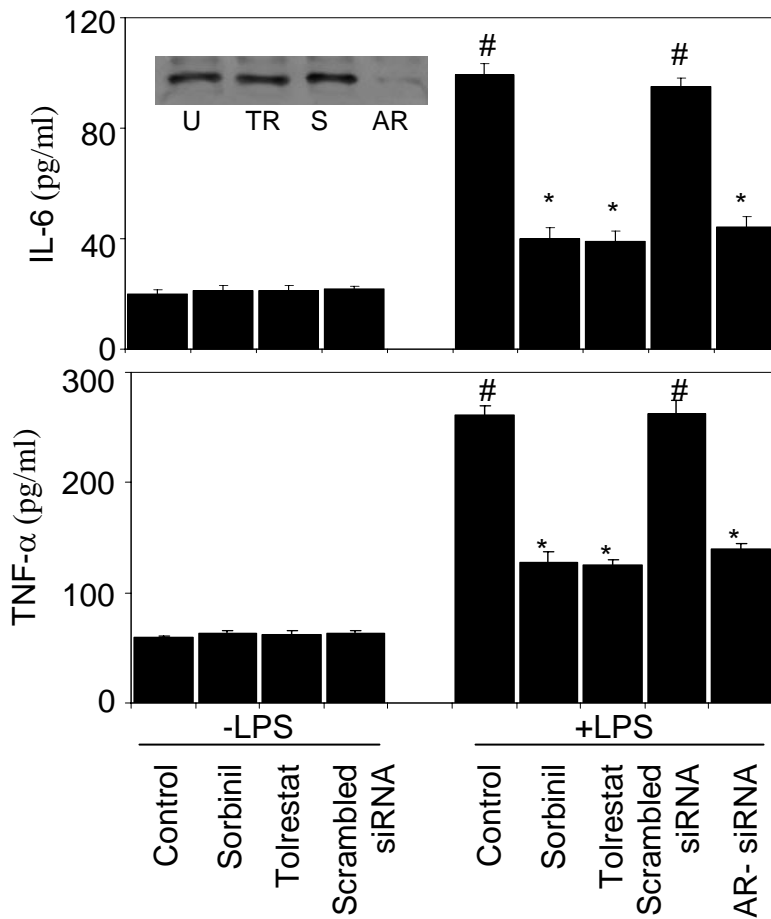
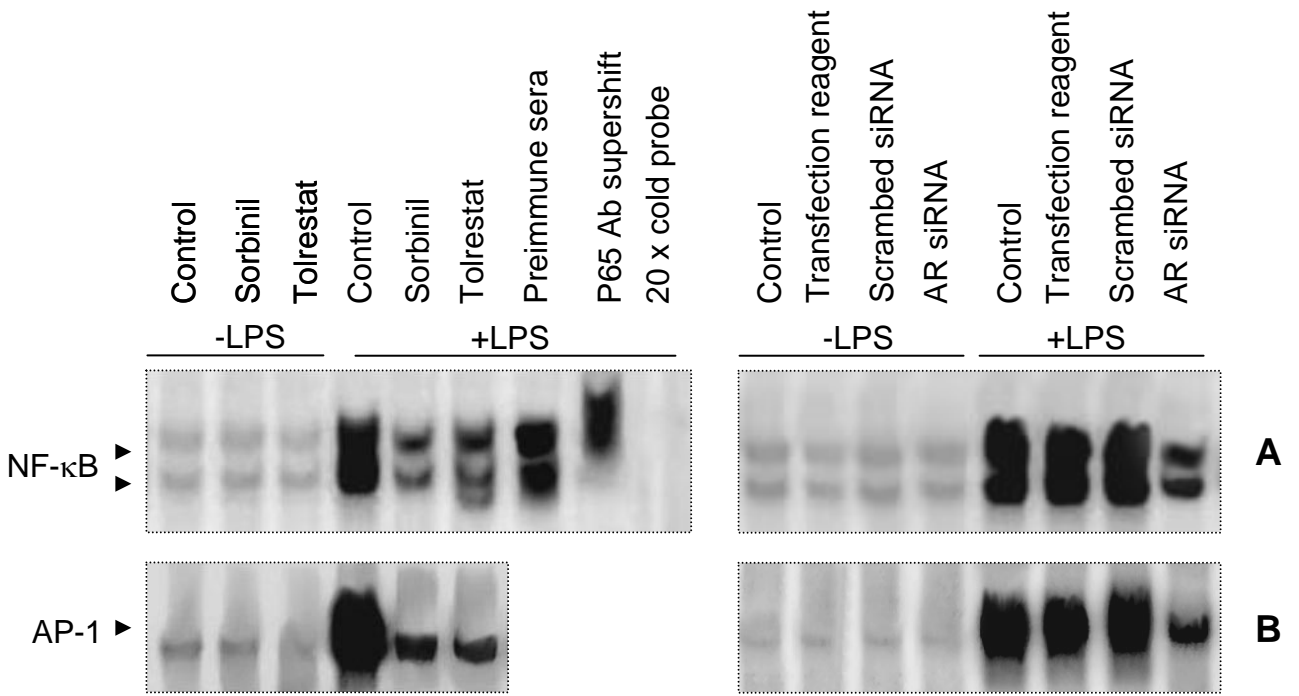
LPS (mg/kg body wt)	LPS alone (Gp1) (% Live mice)	LPS+ARI-2 (Gp3) (% Live mice)	<i>P</i> value*
0	100	100	-
4	100	100	-
8	100	100	-
12	75	87.5	0.99
16	25	66.6	0.0995
20	16.6	41.67	0.37
24	0	33.3	0.093

* Fisher's Exact Test; (number of animals in each group =12)

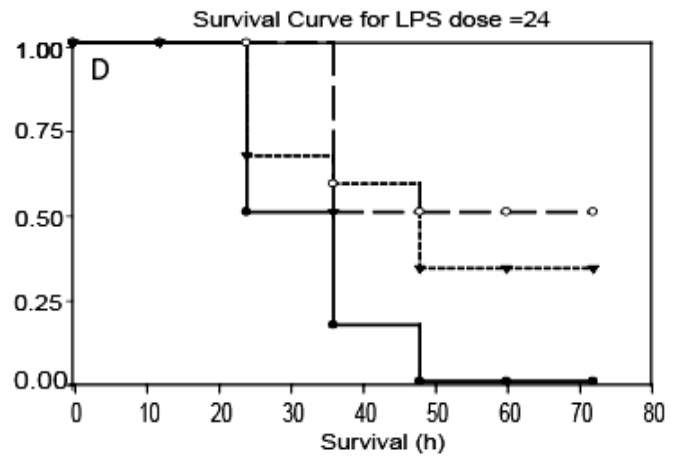
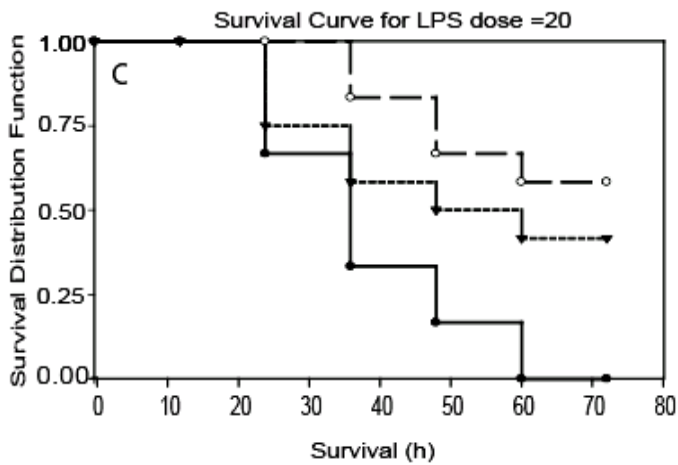
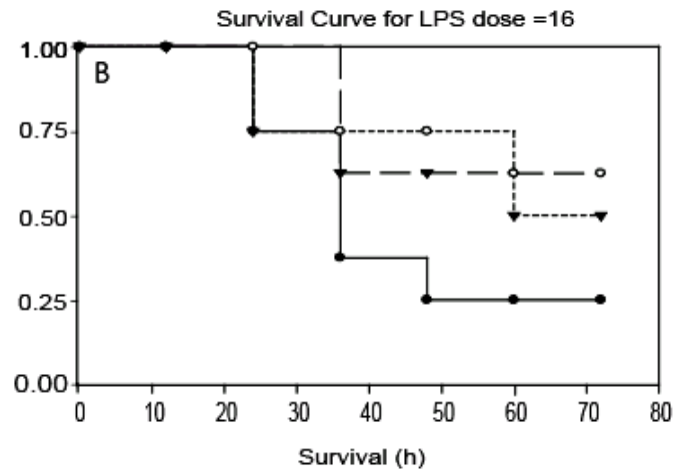
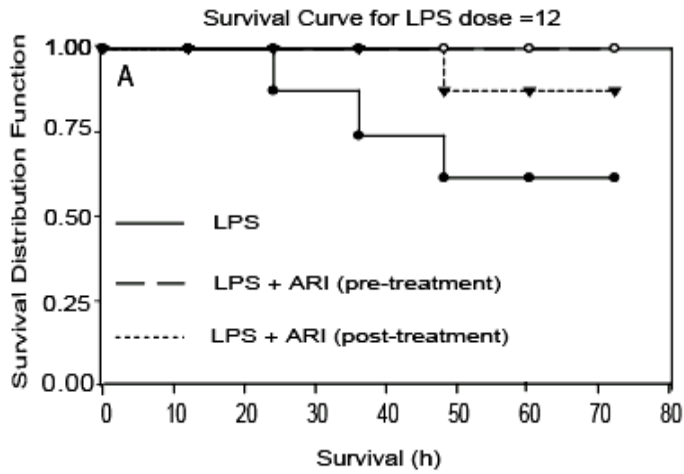
Survival data at 48 h after LPS treatment were analyzed by both chi-square and the Fisher exact test of contingency tables. A) LPS Vs ARI pre-treated groups B) LPS Vs ARI post-treated groups were used to interpret probabilities from the contingency tables using SAS statistical software (SAS Institute INC. 2004. *SAS/STAT[®] 9.1 User's Guide*. Cary, NC: SAS Institute Inc.).



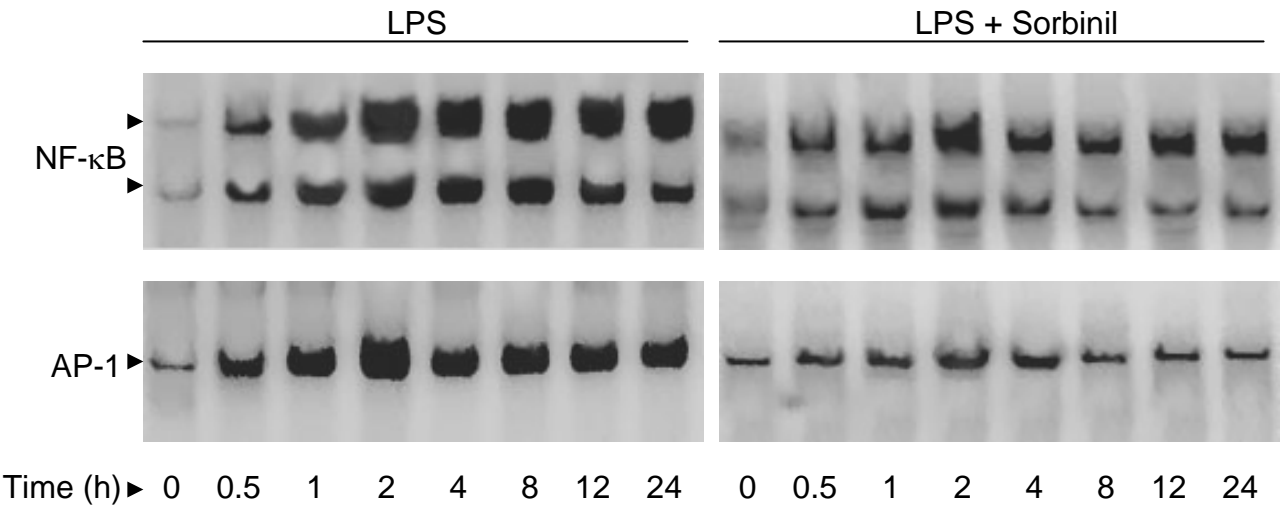
Supplementary Figure -S1



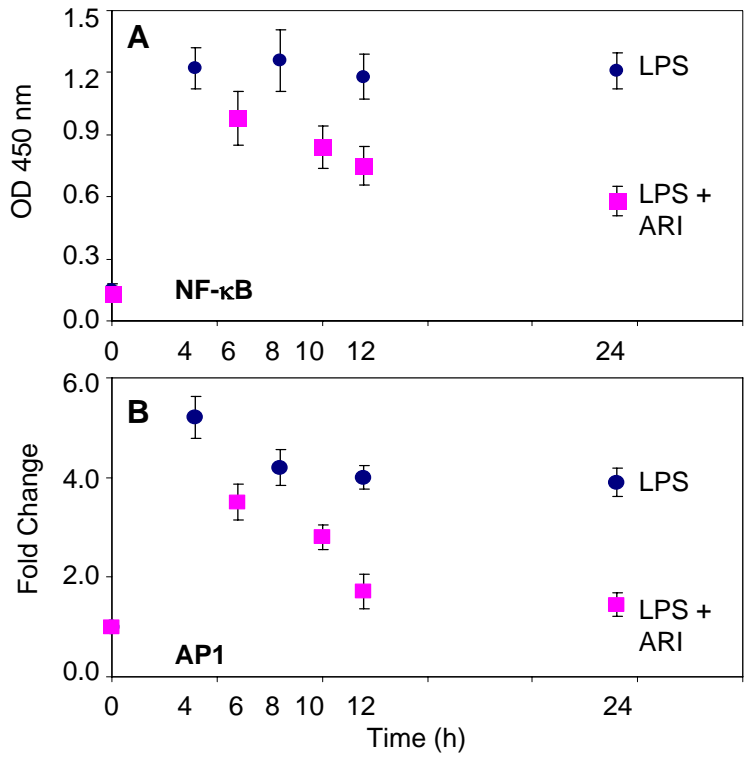
Supplementary Figure –S2



Supplementary Figure –S4



Supplementary Figure –S5



Supplementary Figure –S6