Aldose reductase mediates the lipopolysaccharide –induced release of inflammatory mediators in RAW264.7 murine macrophages

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Abnormal production of inflammatory cytokines and chemokines is a key feature of bacterial endotoxin, lipopolysaccharide (LPS), –induced inflammation and cytotoxicity; however, the mechanisms regulating production of inflammatory markers remain unclear. Herein, we show that inhibition of the aldehyde-metabolizing enzyme aldose reductase (AR; AKR1B3) modulates NF-κB-dependent activation of inflammatory cytokines and chemokines in mouse serum, liver, heart and spleen. Pharmacological inhibition or siRNA ablation of AR prevented the biosynthesis of tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), IL-6, MCP-1 and Cox-2 and PGE2 in LPS- activated RAW264.7 murine macrophages. The AR inhibition or ablation significantly attenuated LPS-induced activation of PKC and PLC, nuclear translocation of NF-κB, phosphorylation and proteolytic degradation of IκB-α in macrophages. Furthermore, treatment of macrophages with 4-hydroxy-trans-2-nonenal (HNE), and cell permeable esters of glutathionyl-4-hydroxynonanal (GS-HNE) and glutathionyl-1,4-dihydroxynonane (GS-DHN) activated NF-κB and PLC/PKC. Pharmacological inhibition or antisense ablation of AR that catalyzes the reduction of GS-HNE to GS-DHN prevented PLC, PKC, IKKα/β and NF-κB activation caused by HNE and GS-HNE, but not by GS-DHN, suggesting that reduced GS-lipid aldehydes, catalyzed by AR propagate LPS- induced production of inflammatory markers. Collectively, these data provide evidence that inhibition of AR may be a significant therapeutic approach in preventing bacterial endotoxin –induced sepsis and tissue damage.

Bacterial lipopolysaccharide (LPS), a proinflammatory endotoxin, is a component of the outer envelope of all gram-negative bacteria (1). When gram-negative bacteria multiply in the host, LPS is released into the circulation, where it is recognized by a variety of circulating cell types, triggering the induction of NF-κB-dependent proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, prostaglandins, and nitric oxide (1-3). Acting in an autocrine and paracrine manner, cytokines and chemokines induce and amplify the host response to bacterial infection (4, 5). However, excessive cytokine release can have deleterious consequences. For example, septic shock triggered by LPS causes production of reactive oxygen species (ROS) and multi-organ dysfunction (2), with myocardial dysfunction being the major cause of morbidity and mortality (6). It has been shown that TNF-α is the earliest cytokine produced in large amounts in response to LPS and that it is the major cause of most of the effects of LPS (7, 8). These studies are supported by the anti-TNF therapy that provides protection against the LPS-induced cytotoxicity (9, 10). Recent studies have shown that heart produces large amounts of cytokines, especially TNF-α,
IL-1β, interferon-gamma (IFN-γ), macrophage-chemoattractant protein-1 (MCP-1) and cyclooxygenase-2 (Cox-2) during septic shock and related pathologies (11, 12). In addition, transgenic mice over-expressing TNF-α readily develop myocardial dysfunction (13). Further, antioxidants such as N-acetyl cysteine and butylated hydroxy toluene (BHT) have been shown to attenuate LPS-induced activation of NF-κB expression of inflammatory cytokines and endotoxemia, indicating that ROS are the obligatory mediators of LPS signaling (14-16). Although these studies have provided possible therapeutic strategies in preventing LPS-induced toxicity, the mechanisms responsible for LPS-induced multi-organ failure remain poorly understood.

Our recent studies show remarkable and unexpected metabolic regulation of TNF-α signaling by the enzyme aldose reductase (AR; AKR1B1 in human, AKR1B4 in rat and AKR1B3 in mouse), a member of aldo-keto reductase superfamily (17-19). AR reduces one of the most abundant and toxic lipid aldehydes, 4-hydroxy-trans-2-nonenal (HNE), to 1, 4-dihydroxynonene (DHN) and its glutathione conjugate, GS-HNE, to GS-DHN (20, 21). We have demonstrated that AR plays a pivotal role in the proliferation of vascular smooth muscle cells (VSMC), apoptosis of vascular endothelial cells (VEC) and restenosis of rat carotid artery (17, 18, 22). Inhibition of AR significantly decreases neointima formation in balloon-injured rat carotid arteries, and also diminishes the in situ activation of NF-κB during restenosis (23). Our recent observations show that AR mediates the mitogenic and cytotoxic signals of cytokines and growth factors (17-19). Inhibition or ablation of AR attenuates TNF-α and growth factor-induced IκB-α phosphorylation and degradation, activation of NF-κB and PKC, proliferation of VSMC, and also apoptosis of VEC and human lens epithelial cells (HLEC) (17-19). Similarly, ablation of AR attenuated the TNF-α-induced expression of adhesion molecules, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) and monocyte adhesion to VEC (18). However, the involvement of AR in inflammatory signals induced by LPS is not known. Therefore, we have investigated the effect of pharmacological inhibition or RNA interference ablation of AR on LPS-induced expression of various cytokines and chemokines and other inflammatory markers such as Cox-2 and PGE2 and examined the possible mechanism of AR mediation in LPS toxicity. Our studies indicate possible therapeutic application of AR inhibitors as anti-inflammatory drugs to treat septic shock induced by gram negative bacterial infections.

**EXPERIMENTAL PROCEDURES**

**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 and Zopolrestat were gifts from Pfizer and Tolrestat was obtained from American Home Products. Normal or phospho-specific antibodies against PLC-β3, PLCγ1, IKK, IκB-α were obtained from Cell Signaling Inc. Mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase antibodies were obtained from Research Diagnostics Inc. protein-HNE antibodies, cyclooxygenase (Cox) activity assay and prostaglandinE2 (PGE2) assay kits were obtained from Cayman chemical company. Colorimetric non-radioactive NF-κB p65 Transcription Factor Assay kit was obtained from Chemicon Laboratories. NF-κB SEAP reporter vector and control vector were obtained from Clontech Laboratories. Lipopolysaccharide (E. coli) and the reagents used in Western blot analysis were obtained from Sigma. All other reagents used were of analytical grade.

**Cell culture and animals**---The Balb/c mice (25-30 g) were obtained from Taconic laboratories and housed in pathogen-free conditions with free access to food and water at the institutional animal care facility. The RAW264.7 macrophage cell lines obtained
from ATCC were grown in DMEM containing 10 % FBS.

RNA interference ablation of AR in macrophages---The ablation of AR mRNA was essentially carried out as described earlier (24). Briefly, RAW264.7 cells were incubated with serum-free medium containing the AR-siRNA (AATCGGTGTCTTCAACTTCAA) or scrambled siRNA (AAAATCTCCCTAAATCATACA; control) to a final concentration of 100 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, and AR expression was determined by measuring AR protein by Western blot analysis using anti-AR antibodies and by measuring AR activity in the total cell lysates (24).

Determination of cytokines levels---The mice were pre-injected with sorbinil (25 mg/Kg body wt, via the intraperitoneal (i.p.) route) or carrier for 24 h followed by LPS (4 μg/kg body wt) injection. At different time intervals the animals were killed and blood and heart tissues were collected. The RAW264.7 cells were pre-incubated with 10 μM of sorbinil, tolrestat or zopolrestat for 24 h followed by incubation with 1 μg/ml of LPS. The cytokines (TNF-α, IL-6, IL-12, and IFN-γ) and chemokine (MCP-1) levels were measured in the mice serum and homogenates of heart, liver and spleen and also in the culture medium of RAW264.7 cells by using BD Biosciences Mouse Inflammation Cytometric Bead Array Kits according to manufacturer’s instructions by FACS automation.

RT-PCR analysis of cytokines---Macrophages were grown in 6 well plates at a density of approximately 3.0x10⁵ cells/ well. The macrophages were serum starved in presence or absence of sorbinil or tolrestat or zopolrestat (10 μM) for 24 h and then stimulated with 1μg/ml LPS. Total RNA from RAW cells was isolated by using RNeasy kit (Qiagen) as per supplier’s instructions. Equal aliquots of RNA (1.0 μg) isolated from each sample were reverse transcribed with Omniscript and Sensiscript reverse transcriptase one-Step RT PCR system with HotStarTaq DNA polymerase (Qiagen) at 55° C for 30 min followed by PCR amplification. The oligonucleotide primer sequences were as follows: 5’-GGCAGGTCTACTTTGGAGTCAATTCG-3’ (sense) and 5’-ACATTGCAGGCTCAGTGAATCCGG-3’ (antisense) for TNF -α, 5’-AAGCTCTCACCTCAATGG-3’ (sense) and 5’-TGCTTGAGAGGTGCTGATGT-3’ (antisense) for IL-1β, 5’-TTCCATCTACGTGCTTTGAC-3’ (sense) and 5’-CTTCATGACTCCAGTAG-3’ (antisense) for IL-6, 5’-AGCGGTGCTGAATCTCAGTATAG-3’ (antisense) for MCP-1, 5’-AGATCCACAAACGGATATT-3’ (sense) and 5’-TCCCTGCAATGGAA-3’ (antisense) for GAPDH. PCR reaction was carried out in a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 95°C for 15 min; 35 cycles of 94°C 30 s, 47- 64°C 30 s, 72°C 1 min, and then 72°C 5 min for final extension. Equal amounts of PCR products were electrophoresed with 2% Agarose-1×TAE gels containing 0.5 μg/ml ethidium bromide.

PGE2 Assay---Macrophages (2x10⁵ cells/well in 6 well plates) were growth -arrested in the serum-free medium without or with AR inhibitors for 24 h followed by incubation with 1 μg/ml of LPS for another 24 h. Similarly AR siRNA or control siRNA-transfected macrophages were serum-starved for 24 h followed by incubation with 1 μg/ml of LPS for another 24 h. The medium was collected from each well and analyzed for PGE2 by using an Enzyme Immuno Assay kit according to the manufacturer's instructions (Cayman Chemical Co.). Briefly, 50 μl of
diluted standard/sample was pipetted into a pre-coated goat polyclonal anti-mouse IgG 96-well plate. Aliquots (50 µl) of PGE2 monoclonal antibody and PGE2 acetylcholine esterase (AChE) conjugate, (PGE2 tracer) were added to each well and allowed to incubate at 4°C for 24 h. After incubation the wells were washed and 200 µl of Ellman’s reagent containing acetylthiocholine and 5, 5'-dithio-bis-(2-nitrobenzoic acid) were added. Samples were read after 60 min at 412 nm with an ELISA reader (Packard).

**Cox activity assay---** Macrophages (2x10^5 cells/well in 6 well plates) were growth arrested in the serum-free medium with or without AR inhibitors for 24 h followed by incubation with 1 µg/ml of LPS for another 24 h. Similarly AR siRNA or control siRNA transfected macrophages were serum-starved for 24 h followed by incubation with 1 µg/ml of LPS for another 24 h. The macrophages were homogenized in cold buffer containing 0.1M Tris-HCl, pH 7.8 and 1mM EDTA and the Cox activity was measured in 96 well plates according to the manufacturer’s instructions (Cayman Chemical Co.). Briefly, 10 µl of standard/sample was incubated in the presence of arachidonic acid and colorimetric substrate, N, N, N, N-tetra methyl-p-phenylenediamine (TMPD) in a total reaction volume of 210 µl. The Cox peroxidase activity was measured colorimetrically by monitoring appearance of oxidized TMPD at 590 nm by using ELISA reader (Packard).

**Determination of PKC Activity---** The membrane bound total PKC activity was measured by using the Promega SignaTECT™ PKC assay system according to the manufacturer’s instructions and as described earlier (17). 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 µCi/µl) and incubated at 30°C for 10 min. The extent of phosphorylation was detected by measuring radioactivity retained on the filter paper by using a scintillation counter.

**Western blot analysis---** An equal amount of macrophage cell extracts were separated on 12% SDS-PAGE, electro-blotted on nitrocellulose membranes and probed with specific antibodies against AR, Cox-1, Cox-2, PLCβ3, PLCγ1, IκB-α, IKK α, β, γ and HNE. 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 and the average changes in fold-intensities were calculated.

**Determination of ROS**---The serum starved macrophages (1.5 x 10⁵ cells/well in a 24-well plate) without or with 10 μM of sorbinil or tolrestat was treated with the ROS-sensitive fluorophore 2', 7'-dichloroﬂuorescin diacetate for 30 min. Subsequently, the macrophages were exposed to LPS (1 μg/ml) for 60 min and fluorescence was measured with a CytoFluorII ﬂuorescence plate reader (PerSeptive Biosystems, Inc., Framingham, MA) at excitation of 485 nm and emission of 528 nm.

**Determination of Intracellular lipid peroxidation**---The lipid peroxidation was determined by measuring total α,β-unsaturated aldehyde (26) levels in RAW264.7 cells (1 x 10⁶ cells/well) treated without or with LPS in the absence and presence of sorbinil. The aldehydes were quantified colorimetrically by using a lipid peroxidation kit (Bioxytech LPO-586™) obtained from Oxford Biomedical Research, Oxford, MI, as per the supplier’s instructions. Briefly, the determination is based on the reaction of the chromogenic reagent, methanesulfonic acid with α,β-unsaturated aldehydes such as HNE at 45 °C. One molecule of aldehyde reacts with two molecules of reagent to yield a stable chromophore with maximal absorbance at 586 nm.

**Preparation of GS-aldehyde esters**---HNE was synthesized as described previously (20). The conjugate of glutathione ethyl ester with HNE (GS-HNE-ester) was prepared as described (25). Briefly, 1 µmol of [4-³H] HNE (55000 cpm/nmol) was incubated with 5 µmol of GSH ethyl ester in 0.1 M potassium phosphate, pH 7.0 for 1 h at room temperature. The reaction was monitored by following the decrease in absorbance at 224 nm. The GS-HNE-ester was purified by reverse phase HPLC. The reduced form of the esterified glutathione-HNE conjugate (GS-DHN-ester) was prepared by incubating 100 nmol of GS-HNE-ester with 300 nmol of NADPH and 100 µg AR in 0.1 M potassium phosphate, pH 6.0 for 3 h at 37°C. The reaction was monitored by following the consumption of NADPH at 340 nm. The GS-DHN-ester was separated from GS-HNE-ester by reverse phase HPLC by using a Varian reverse phase ODS C₁₈ column pre-equilibrated with 0.1% aqueous-trifluoroacetic acid (TFA). The compounds were eluted using a gradient consisting of solvent A (0.1% aqueous TFA) and solvent B (100% acetonitrile) at a flow rate of 1 ml/min. The gradient was established such that solvent B reached 24% in 20 min, 26% in 30 min, and was held at this value for 10 min. In the next 10 min solvent B reached 60%, and in an additional 5 min it reached 100% where it was held for 10 min. Chemical identities of the GS-HNE and GS-DHN-esters were established by electrospray ionization mass spectrometry (ESI/MS) as described before (25). ESI/MS of GS-HNE-ester and GS-DHN-ester show an m/z values of 492.2 and 494.2 (data not shown), respectively.

**Statistical analysis**---Data are presented as mean ± SEM and the P values were determined using the unpaired student’s t-test.

**RESULTS**

**Effect of AR inhibition on LPS-induced cytokine production in mice serum, spleen, heart and liver tissues**---A single intraperitoneal injection of LPS in mice caused 3-, 4-, 7.5- and 1.5-fold increase in serum IL-12, TNF-α, IL-6 and MCP-1 levels, respectively on day 1, which gradually decreased to basal levels on day 7 (Table-1). In sorbinil+LPS -treated mice the serum levels of IL-12, TNF-α, IL-6 and MCP-1 were only slightly higher compared to basal levels on day 1 and returned to basal levels on day 7. Similarly, as shown in Table -2, LPS caused
2-, 2.5-, 1.5- and 2-fold induction of heart IL-12, TNF-α, IL-6 and MCP-1, respectively, on day 1, which gradually decreased but remained higher than basal levels on day 7. In spleen, LPS caused 2-, 4-, 5- and 1.5-fold induction of IL-12, TNF-α, IL-6 and MCP-1, respectively on day 1, which significantly decreased on day 7. In liver, LPS caused 2-, 2.5-, 3- and 2-fold induction of IL-12, TNF-α, IL-6 and MCP-1, respectively on day 1, which gradually decreased to basal levels on day 7. However in sorbinil+LPS treated animals the heart, spleen and liver levels of IL-12, TNF-α, IL-6 and MCP-1 were only slightly higher than basal levels on day 1 and on day 7 these levels were at or below the basal levels, suggesting that inhibition of AR could prevent LPS-induced production of cytokines and chemokines in mice.

Inhibition of AR prevents LPS-induced cytokine production in RAW264.7 macrophages—To examine the mechanism of AR-mediated regulation of LPS-induced production of cytokines and chemokines in mice, and to exclude the possible non-specific inhibition of other enzymes by sorbinil, we systematically examined the effect of pharmacological inhibition of AR by three structurally different inhibitors, sorbinil, tolrestat and zopolrestat and also by ablating AR message by RNA interference. We first determined the effect of AR inhibition/ablation on LPS-induced production of cytokines and chemokines in RAW 264.7 macrophages. As shown in Fig.1 A-D (left panels); incubation of RAW264.7 macrophages with LPS for 16 h caused 28-, 11-, 50- and 5-fold increase of TNF-α, IL-1β, IL-6 and MCP-1 levels, respectively in the culture medium (Fig 1, A-D, right panels). Transfection of cells with AR-siRNA but not control siRNA significantly (80-90%), prevented LPS-induced increase in the levels of TNF-α, IL-1β, IL-6, and MCP-1. However, AR ablation alone did not change the basal levels of these inflammatory cytokines and chemokines in macrophages. To confirm results obtained with bead array system, we have performed RT-PCR analysis. Incubation of macrophages with LPS for 4 h caused significant 3.5-, 6-, 5- and 7-fold increase in TNF-α, IL-β, IL-6, IL-1β, and MCP-1 mRNA levels, respectively (Fig. 3). Inhibition of AR by three structurally distinct inhibitors significantly (80-90%) prevented LPS-induced increase in the mRNA levels of TNF-α, IL-6, IL-1β, and MCP-1 suggesting the involvement of AR in the LPS-mediated increase in inflammatory signals.

AR inhibition/ablation attenuates LPS-induced Cox-2 expression and PGE2 production in RAW264.7 cells—LPS caused a 17-fold increase in the biosynthesis of PGE2 as compared to untreated cells. However when the macrophages were challenged with LPS in the presence of AR inhibitors or AR ablation only 4 fold increase in the biosynthesis of PGE2 was observed. AR inhibitors alone did not alter the basal biosynthesis of PGE2 (Fig.4A and 4B). Since the biosynthesis of PGE2 is catalyzed by Cox-1 and Cox-2 enzymes, we next measured the effect of AR
inhibition and ablation on LPS-induced Cox activities. LPS increased Cox activity by 9-fold and inhibition or ablation of AR significantly (>80%) prevented the LPS-induced increase of Cox activity (Fig. 5A and 5B). Since the activity of Cox is contributed by Cox-1 (constitutive) and Cox-2 (inducible), we next measured the effect of AR inhibition on LPS-induced expression of these proteins in macrophages. As shown in Fig 5C, the levels of Cox-1 were not affected by LPS but Cox-2 protein increased by ~3 fold (Fig. 5D). The LPS-induced increase in Cox-2 protein was abolished by AR inhibition.

**AR inhibition/ablation prevents LPS-induced activation of NF-κB in macrophages**— Activation of redox-sensitive transcription factor, NF-κB transcribes the genes necessary for induction of inflammatory Cox-2, cytokines, and chemokines. We therefore examined the effect of inhibition and ablation of AR on LPS-induced NF-κB activation in RAW264.7 cells. Within 2 h of LPS addition to macrophages ~10 fold activation of NF-κB was observed and the increase was significantly attenuated by AR inhibitors as well as antisense ablation of AR (Fig. 6 A and B). However, AR inhibitors as well as AR ablation had no effect on basal NF-κB activity in macrophages. The inhibitory effect of AR on NF-κB activity was further analyzed in macrophages transfected with NF-κB SEAP reporter plasmid by monitoring the SEAP activity in response to LPS challenge. AR inhibition or ablation also decreased the LPS-dependent activation of NF-κB SEAP activity in macrophages transfected with pNF-κB SEAP plasmid but not in control pTALSEAP plasmid (Fig. 6C and D). Modulation of NF-κB by AR inhibitors was further supported by immunoblot analysis of p65 in cytoplasmic and nuclear extracts of LPS and LPS+sorbinil-treated macrophages. As shown in Fig. 7A, LPS caused translocation of p65 from cytoplasm to the nuclei within 10 min of LPS challenge. The LPS-induced nuclear localization of p65 was prevented by pretreating the cells with sorbinil suggesting that AR inhibition acts upstream to nuclear translocation of NF-κB. This conclusion is further supported by the modulation of LPS-induced phosphorylation as well as degradation of IκB-α by AR inhibition (Fig. 7B).

**Inhibition of IKK activities by ARI**— Macrophages were stimulated with LPS in the presence or absence of AR inhibitor, sorbinil and whole cell extracts were tested for phosphorylation status of IKKs by using phospho-specific antibodies for IKK, which recognizes phosphorylation of both α and β forms of IKK. LPS significantly increased the phosphorylation of IKKα/β and AR inhibition prevented it (Fig. 7C). However, LPS alone or LPS+sorbinil did not alter the expression of IKK-α, β and γ isozymes (Fig. 7D). These observations suggest that AR inhibition prevents NF-κB DNA binding as well as its transcriotional activity by inhibiting phosphorylation of IKKα/β activities which indicates the possible involvement of upstream kinases in the activation of IKK.

**The inhibition/ablation of AR prevents LPS-induced PKC activity in RAW264.7 macrophages**— Inhibition or ablation of AR inhibited the LPS-induced phosphorylation of total membrane-bound PKC (Fig. 8A and 8B). Since PLC is upstream to PKC, we examined the effect of AR inhibition on LPS-induced PLC activity. The maximum activities of PLC-β3 and γ1 were observed at 5 and 20 min respectively, after incubation of macrophages with LPS, which were inhibited by AR inhibition (Fig. 8C and 8D). These observations indicate that the inhibitory mechanism of AR inhibitors on NF-κB activation may be via the PLC-PKC pathway.

**Inhibition of AR prevents LPS-induced ROS production**— Since LPS is known to increase ROS, we next examined the effect of AR inhibition on LPS-induced generation of ROS. As shown in Fig. 9A, treatment of RAW264.7 cells with LPS caused a significant increase in ROS levels in 60 min and AR inhibition prevented it. Since ROS cause peroxidation of membrane lipids
resulting in the formation of lipid aldehydes such as 4-hydroxy-trans-2-nonenol (HNE) which could readily conjugate with glutathione (GSH) and both HNE and GS-HNE can be reduced by AR, we investigated the effect of AR inhibition on LPS-induced lipid peroxidation caused by LPS in macrophages. As expected, LPS increased the levels of α, β-unsaturated aldehydes and protein-HNE adducts by nearly 3-fold within 6 h (Fig. 9B and 9C) and inhibition of AR slightly increased the levels of α,β-unsaturated aldehydes and protein-HNE adducts.

Reduced glutathione-aldehyde conjugates catalyzed by AR propagate LPS-signals in macrophages--- Since the host response to LPS is known to be mediated by ROS (14-16), we asked if AR-mediated reduction of toxic lipid aldehydes and their glutathione conjugates such as GS-DHN could activate the inflammatory cascade? Treatment of RAW264.7 macrophages with HNE (1 μM) and cell permeable esters of GS-HNE or GS-DHN (1 μM) resulted in the phosphorylation of IKK-α/β and activation of NF-κB (Fig. 11A and 10A). Inhibition or ablation of AR significantly blunted the effects of HNE/GS-HNE on IKK-α/β phosphorylation and NF-κB activation but had no effect on the ability of GS-DHN, the already reduced form of GS-HNE, to activate NF-κB (Fig. 10A & 11A). This suggested that GS-DHN is sufficient for NF-κB activation and may be involved in IKK-α/β phosphorylation.

To determine if GS-DHN serves as a cellular sensor of ROS-induced insults, we examined its effects on the phosphorylation events upstream of IKK/NF-κB activation in RAW264.7 macrophages. After GS-DHN challenge, the activity of PKC increased by ~2.5 fold within 60 min (Fig. 10B). GS-DHN also induced the phosphorylation of PLC-β3 and PLC-γ1 (Fig. 11B & C), which activated PKC, but had no effect on the total PLC protein (not shown). As expected, HNE and GS-HNE had similar effects on the phosphorylation of the kinases upstream of NF-κB (Fig. 11A to C and 10B). However, pharmacologic inhibition or siRNA-mediated ablation of AR significantly decreased the HNE and GS-HNE-induced phosphorylation of PLC, PKC, and IKK, but had no effect on GS-DHN-initiated phosphorylation of PLC and its downstream kinases. These findings suggest that glutathione-lipid alcohol (such as GS-DHN) formed by the reduction of glutathione–lipid aldehyde (such as GS-HNE) catalyzed by AR could be an obligatory mediator of LPS-induced inflammation.

DISCUSSION

To the best of our knowledge, the present study is the first to demonstrate that inhibition of AR significantly improves LPS–induced cytotoxicity leading to formation of inflammatory cytokines. LPS, an endotoxin found in the outer membrane of Gram-negative bacteria, is a major trigger of septic shock (1-3), which is, in part, a consequence of the host’s response to overwhelming bacterial infection. Sepsis is characterized by microvascular thrombosis, decreased organ perfusion, and organ ischemia, which leads to multi-organ dysfunction and death (2, 26, 27). Innate immune cells such as macrophages recognize the presence of invading bacteria, and initiate the host response by releasing cytokines and chemokines (6). When cytokines in the inflammatory cells are present in effective concentrations, pathogens are removed without adverse consequences; however, excessive amounts of inflammatory cytokines lead to septic shock and death (2, 27, 28). A decrease in cardiac muscle contractility is the major cause of mortality and morbidity in sepsis (8, 9). Despite substantial advances in antimicrobial therapy, the mortality in severe sepsis continues to be ~40%, reflecting the limited therapeutic options (29).

The mechanisms underlying the multiorgan failure, especially heart failure that occurs during septic shock have been the subject of intense investigation. It is well known that activation of redox-sensitive transcription factors such as NF-κB and AP-1 are involved in the pathologies associated with
LPS-induced sepsis (30-32). However, the precise mechanisms of LPS-signaling leading to the activation of these transcription factors are not known. Even though multiple studies suggest that anti-oxidant therapy prevents LPS-induced septic shock (14-16), the mechanisms by which ROS mediate inflammatory signals leading to NF-κB activation are not clearly understood. Since lipid peroxidation initiated by ROS generates toxic aldehydes that conjugate with glutathione and can be reduced by AR (33), we systematically investigated the effect of inhibiting LPS-induced cytotoxicity without or with inhibition or ablation of AR in macrophages. Inhibition of AR prevented the LPS-induced activation of NF-κB and release of proinflammatory cytokines, indicating that such inhibitors could be used therapeutically to treat bacterial sepsis. Similar results with AR ablation indicate that pharmacological inhibition is equally efficient.

Rodents have been used extensively as an experimental animal model for LPS-induced septic shock (34-36), where increased levels of serum cytokines, in particular TNF-α are observed (7, 8). Therefore, we investigated the effect of AR inhibition on LPS-induced serum cytokine and chemokine levels using a mouse model of sepsis. Consistent with the results obtained in cultured macrophages, severely elevated levels of serum cytokines subsequent to LPS challenge were markedly suppressed by AR inhibition in vivo, suggesting an anti-inflammatory role for AR inhibitors in mice. Various reports from experimental models of endotoxin challenge and patients in septic shock have shown multi-organ dysfunction (2, 37, 38). The major factors that influence multi-organ dysfunction include cytokines such as TNF-α, IL-1β, and IL-6, and other inflammatory mediators such as nitric oxide, prostaglandins, Cox-2 and lipid metabolites (11, 12). The cytokines are generally considered to be circulating molecules, but tissues such as heart, liver and spleen also synthesize TNF-α, IL-1β, and IL-6 (36, 37). In fact, increased local production of cytokines in the heart has been strongly implicated in myocardial dysfunction observed during various pathological conditions (11, 12). Increased synthesis and autocrine secretion of inflammatory cytokines may have local consequences on cardiac contractile protein mRNA expression. For example, IL-1β activates whereas IL-6 represses contractile protein genes (39, 40). Whether the autocrine production of cytokines, in addition to cytokines produced as a consequence of the systemic inflammatory response, contributes to the multi-organ dysfunction in sepsis is unknown. Since, serum levels of LPS-induced cytokines are inhibited by AR inhibition; we investigated the effect of AR inhibition on cardiac, hepatic and splenic levels of cytokines. Indeed, inhibition of AR resulted in the inhibition of cytokines and other inflammatory factors generation locally in the tissues indicating a major role of AR in the regulation of LPS-induced multi-organ dysfunction. Taken together, this study is the first to demonstrate that AR inhibition prevents endotoxin-induced organ dysfunction.

Prevention of LPS-induced increase in the activation of NF-κB and activation of protein kinases upstream of NF-κB such as IKK and PKC by AR inhibition or ablation suggests that AR inhibition could attenuate LPS-induced inflammation by disrupting a signaling cascade upstream of PKC/PLC, which causes synthesis of cytokines and chemokines via NF-κB. These results are in agreement with our earlier observations that inhibition of AR prevents cytokine and hyperglycemia–induced NF-κB signaling in VSMC, VEC and HLEC (17-19, 23, 24). Even though AR inhibition attenuated phosphorylation of PLC, it was not clear how AR could mediate ROS- signals. Since, we have shown earlier that AR reduces lipid aldehydes and their conjugates with glutathione to corresponding alcohols (20, 21), it was possible that the reduced form of lipid aldehydes or their glutathione conjugates act as signaling intermediates. To investigate this possibility, we examined the effect of AR inhibition/ablation on HNE, and cell
permeable esters of GS-HNE and GS-DHN–induced cell signaling. Our observations that AR inhibition/ablation prevented HNE and GS-HNE-ester induced signals and subsequent activation of NF-κB /PKC/IKK/PLC, but GS-DHN-ester signaling was resistant to AR inhibition, suggest a novel role for a reduced glutathione -lipid aldehyde conjugate (such as GS-DHN) as an obligatory mediator of ROS-induced cytotoxicity. Our recent study indicates that GS-HNE and GS-DHN-esters readily enter in the cells where esterases cleave off ester group (25).

In summary, this study shows that AR-catalyzed reduction of lipid aldehydes produced by ROS in response to endotoxin is necessary for activation of the signaling cascade leading to NF-κB nuclear translocation and increased production of inflammatory cytokines. The observation that inhibition of AR dramatically attenuates LPS-induced cytokine production in vitro and in vivo suggests that modulation of lipid aldehyde reduction could provide a cellular approach for preventing the maladaptive host response to bacterial infections and other NF-κB dependent cellular events. Specific inhibitors of AR could be used for short term therapy of severe bacterial toxin-induced sepsis and associated inflammatory processes.

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Keywords: Aldose reductase, inflammation, lipid aldehydes, LPS, NF-kappaB

The abbreviations used are: AR, aldose reductase; ARI, aldose reductase inhibitor; Cox, cyclooxygenase; DHN, 1,4-dihydroxynonene; HNE, 4-hydroxy-trans-2-nonenal; GSH, glutathione; GS-HNE, glutathionyl-4-hydroxynonanal; GS-DHN, glutathionyl-1,4-dihydroxynonane; LPS, lipopolysaccharide; PGE2, prostaglandin-E2; NF-κB, nuclear factor kappa binding protein; PKC, Protein kinase C; PLC, Phospholipase C; SEAP, Secretary alkaline phosphatase; siRNA, small interfering RNA; MCP-1, macrophage chemoattractant protein-1.

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Figure legends:

Fig. 1: Effect of AR inhibition/ablation on LPS-induced production of inflammatory cytokines in RAW264.7 macrophages. A-D) Cells were growth arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without indicated AR inhibitors (10 μM, left panels) or transfected with control or AR siRNA oligonucleotides (right panels), and challenged with LPS (1 μg/ml). The cytokine and chemokine levels were measured at 16 h in the culture media of macrophages by using BD Biosciences Mouse Inflammation Cytometric Bead array kit as described in the Experimental Procedures. All the data are expressed as Mean ± SEM (N = 4). *P < 0.001 as compared to LPS-treated cells #P < 0.001 control cells.

Fig. 2: Ablation of AR by RNA interference in RAW264.7 macrophages. The serum-starved RAW264.7 cells were transfected with double-stranded AR-specific siRNA as described in the Experimental Procedures. A) Aldose reductase activity determined using DL-glyceraldehyde and NADPH as substrates. Bars represent means ± SE (n = 4); *P < 0.001 vs. control siRNA transfected cells. B) and C), Western blots analysis of RAW264.7 cell extracts developed using anti–aldose reductase or anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies, respectively.

Fig. 3: Effect of AR inhibition on LPS-induced mRNA levels of inflammatory cytokines in RAW264.7 macrophages. Cells were growth -arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without indicated AR inhibitors and challenged with LPS (1 μg/ml). The total RNA was isolated at 4 h and RT-PCR analysis was carried out using specific primers for indicated cytokines as described in the Experimental Procedures. Equal amounts of PCR products were electrophoresed with 2% Agarose-1×TAE gels containing ethidium bromide. RT-PCR analysis with GADPH served as control.

Fig. 4: Effect of AR inhibition/ablation on LPS-induced production of PGE2 in RAW264.7 macrophages. Cells were growth -arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without indicated AR inhibition/ablation and challenged with LPS. The PGE2 released in the culture medium was determined by using monoclonal Enzyme Immuno Assay kit. AR inhibition by A) inhibitors and B) siRNA. All the data are expressed as Mean ± SEM (N = 4). *P < 0.001 as compared to LPS-treated cells #P < 0.001 control cells. (UT= untransfected; TR= transfection reagent)

Fig. 5: Effect of AR inhibition/ablation on LPS-induced activation of Cox-2 in RAW264.7 macrophages. Cells were growth -arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without indicated AR inhibition/ablation and challenged with LPS, AR inhibition by A) inhibitors and B) siRNA. The Cox activity was determined as described in the Experimental Procedures. C-E) Western blots were developed using antibodies aginst C) Cox-1, D) Cox-2 and E) anti-GAPDH antibodies. The antibody binding was detected by enhanced pico chemiluminescence (Pierce). All the data are expressed as Mean ± SEM (N = 4). *P < 0.001 as compared to LPS-treated cells #P < 0.001 control cells. (UT= untransfected; TR= transfection reagent)

Fig. 6: Effect of AR inhibition/ablation on LPS-induced activation of NF-κB in RAW264.7 macrophages. Cells were growth -arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without indicated AR inhibition/ablation and challenged
with LPS. A & C) AR inhibitors and B & D) siRNA ablation. The NF-κB activity was measured by using A, B) p65 Transcription Factor Assay kit and C, D) secretary alkaline phosphatase (SEAP) reporter assay as described in the Experimental Procedures. The data are expressed as Mean ± SEM (N = 4). *P < 0.001 as compared to LPS-treated cells 

Fig. 7: Effect of AR inhibition on LPS-induced activation of IKK-α/β and phosphorylation/degradation of IκB-α. Cells were growth -arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without sorbinil and challenged with LPS for indicated time periods. The pooled cytoplasmic and nuclear extracts from 3 independent experiments were subjected to SDS-PAGE and Western blots were developed using antibodies against A) p65 in cytoplasmic (CE) and nuclear extracts (NE); B) top) phospho-IκB-α specific and B) bottom) unphosphorylated-IκB-α; C) phospho-IKK-α/β; D) unphosphorylated-IKK-α, β and γ; and E) anti-GAPDH. The antibody binding was detected by enhanced pico chemiluminescence.

Fig. 8: Effect of AR inhibition on LPS-induced activation of PKC and PLC in macrophages. Cells were growth -arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without indicated AR inhibition/ablation and challenged with LPS. AR inhibition by A) inhibitors and B) siRNA. The membrane-bound PKC activity was determined using Promega SignaTect™ total PKC assay system. All the data are expressed as Mean ± SEM (N = 4). *P < 0.001 as compared to LPS-treated cells 

Fig. 9: Effect of AR inhibition on LPS-induced oxidative stress in macrophages: The serum starved macrophages with or without sorbinil or tolrestat were A) treated with 2', 7'-dichlorofluorescein diacetate for 30 min followed by LPS for 60 min. The levels of ROS plotted as relative fluorescence units. B and C), incubated with LPS at indicated times. The levels of A) α,β-unsaturated aldehydes and B) protein-HNE adducts were measured as described in the Experimental Procedures. For Protein-HNE adducts the densitometric units (Arbitrary units) are plotted. All the data are expressed as Mean ± SEM (N = 3). *P < 0.01 as compared to LPS-treated cells.

Fig. 10. Effect of AR inhibition/ablation on lipid aldehyde–induced NF-κB and PKC in RAW264.7 cells. Cells were growth -arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without sorbinil or were transfected with control or AR siRNA oligonucleotides. The cells were incubated with HNE, GS-HNE-ester, and GS-DHN-ester and A) NF-κB and B) PKC were determined as described in the Experimental Procedures. Values are means ± SEM (N = 4). **P < 0.001, *P < 0.01 versus HNE- or GS-HNE-, treated cells.

Fig. 11. Effect of AR inhibition/ablation on lipid aldehyde–induced IKK and PLC activities in RAW264.7 cells. Cells were growth -arrested in Dulbecco’s modified Eagle’s medium containing 0.1% serum with or without sorbinil or were transfected with control or AR siRNA oligonucleotides. The cells were incubated with 1 μM each of HNE, GS-HNE-
ester, or GS-DHN-ester for indicated time points. The pooled extracts from 3 independent experiments were subjected to SDS-PAGE and Western blots were developed using antibodies against A) phospho-ΙΚΚα/β, B) phospho-PLC-β1 C) phospho-PLC-γ1. The antibody binding was detected by enhanced pico chemiluminescence.
Table-1: Effect of AR inhibitor on LPS-induced cytokine and chemokine production in mice serum

| days | **IL-12** (pg/mg protein) | **TNF** (pg/mg protein) | **IL-6** (pg/mg protein) | **MCP-1** (pg/mg protein) |
|------|--------------------------|-------------------------|--------------------------|--------------------------|
|      | 1 | 3 | 7 | 1 | 3 | 7 | 1 | 3 | 7 | 1 | 3 | 7 |
| Control | 27.34± | 26.72± | 26.09± | 24.2± | 22.9± | 25.78± | 8.92± | 9.16± | 9.89± | 33.7± | 38.37± | 49.11± |
| Sorbinil | 29.37± | 26.97± | 25.43± | 28.3± | 23.32± | 25.9± | 10.2± | 9.3± | 8.96± | 29.54± | 32.29± | 32.21± |
| LPS | 107.55± | 57.9± | 31.34± | 145.4± | 63.74± | 34.89± | 57.76± | 49.36± | 45.67± | 57.14± | 87.52± | 189.33± |
| LPS+sorbinil | 43.4± | 23.23± | 18.0± | 43.4± | 27.9± | 17.95± | 15.81± | 11.31± | 10.62± | 29.32± | 25.5± | 37.29± |

The Balb/c mice (25 g) were pre-injected i.p. with sorbinil (25 mg/kg body wt/day) for 2 days or carrier followed by single i.p. injection of LPS (4 μg/kg body wt). Sorbinil injections were given at every 24 h after LPS injection. The mice were killed at 1, 3 and 7 days after LPS-injection and serum was prepared from the blood. The cytokines/chemokines levels were measured in the serum by using BD biosciences Mouse Inflammation Cytometric bead array kit. All data are expressed as mean ± S.E.M. * P value < 0.05, ** P value < 0.001 LPS+sorbinil compared to LPS group.  # P value < 0.05, ## P value < 0.001 as control group is compared to LPS group.
Table-2: Effect of AR inhibitor on LPS-induced cytokine and chemokine production in mice heart, spleen and liver tissue homogenates.

| Cytokines | IL-12 (pg/mg protein) | TNF (pg/mg protein) | IL-6 (pg/mg protein) | MCP-1 (pg/mg protein) |
|-----------|-----------------------|---------------------|----------------------|----------------------|
| days      | 1 3 7                 | 1 3 7               | 1 3 7                | 1 3 7                |
| Control   | 34.2 ± 24.8 ± 32.37 ± | 39.1 ± 33.9 ± 38.8 ± | 9.14 ± 9.27 ± 6.9 ±  | 121.59 ± 106.9 ± 106.5 ± |
| LPS       | 7.6 1.26 2.89         | 5.8 10.4 9.8        | 1.4 0.24 2.3         | 12.2 19.3 5.6       |
| Sorbinil  | 31.5 ± 24.23 ± 32.4 ± | 32.23 ± 29.1 ± 32.6 ± | 9.6 ± 8.6 ± 8.6 ±    | 109.1 ± 98.1 ± 91.35 ± |
| LPS       | 11.76 5.26 10.4       | 11.3 8.3 8.0        | 2.8 1.8 2.3          | 17.1 7.1 22         |
| LPS+      | 69.8 ± 50.26 ± 48.8 ± | 92.1 ± 62.0 ± 53.6 ± | 21.3 ± 13.3 ± 10.8 ± | 220.79 ± 128.9 ± 108.6 ± |
| sorbinil  | 12.2# 15.2### 10.9### | 12.6### 11.0### 6.7### | 4.6### 2.3# 3.7###  | 27.46### 18.6 3.78  |
| LPS       | 35.1 ± 25.7 ± 42.7 ± | 37.4 ± 27.0 ± 40.9 ± | 7.8 ± 8.9 ± 6.7 ±    | 96.8 ± 83.2 ± 99.8 ± |
| sorbinil  | 8.21* 5.47** 7.57     | 7.16** 3.1** 7.56*  | 2.32** 0.23* 1.4*    | 15.7** 5.9* 12.4*   |
| Control   | 43.6 ± 37.6 ± 32.5 ± | 142.6 ± 144.6 ± 159.8 ± | 8.95 ± 9.55 ± 9.50±  | 62.12 ± 55.7 ± 66.7 ± |
| LPS       | 8.2 3.8 12.25         | 21.5 9.55 5.7       | 1.81 2.11 3.21       | 10.31 22.1 4.8      |
| Sorbinil  | 36.5 ± 39.9 ± 35.4 ± | 136.7 ± 155.26 ± 150.9 ± | 12.62 ± 13.46 ± 11.76±  | 67.14 ± 57.4 ± 60.9 ± |
| LPS       | 9.6 8.9 3.75          | 34.26 ± 30.46 ± 5.9 | 2.3 3.8 2.89         | 16.2 6.92 3.97      |
| LPS+      | 10.27# 13.7# 12.35#  | 540.7 ± 242.0 ± 235 ± | 43.9 ± 29 ± 27.78±   | 108.6 ± 96.6 ± 80.8 |
| sorbinil  | 36.36 ± 39.6 ± 19.68 ± | 189.71 ± 171.1 ± 180 ± | 12.4 ± 15.2 ± 8.2 ±  | 69.6 ± 63.2 ± 50.36 ± |
| LPS       | 12.5** 15.2* 10.12*  | 15.6** 15.2* 14*    | 6.45** 3.23* 2.4**   | 18.1* 8.26* 24.4*   |
| Control   | 55.3 ± 54.5 ± 53.56 ± | 28.89 ± 32.82 ± 30.1 | 2.83 ± 3.96 ± 3.7 ±  | 42.28 ± 38.58 ± 37.22 ± |
| LPS       | 12.28 14.28 13.25     | 8.2 6.14 ±4.78      | 1.01 0.5 2.0         | 8.6 3.96 5.65       |
| Sorbinil  | 50.4 ± 65.3 ± 51.35 ± | 29.62 ± 27.36 ± 29.5 ± | 3.31 ± 4.7 ± 8.6 ±   | 34.2 ± 34.72 ± 34.56 ± |
| LPS       | 15 5.3 13.5           | 4 8.5 1.2           | 0.25 1.0 2.8         | 6.58 2.8 1.5        |
| LPS+      | 122.3 ± 94.6 ± 101.56 | 78.2 ± 57.56 ± 38.49 ± | 9.80 ± 9.76 ± 10.6 ± | 88.4 ± 53.14 ± 42.6 ± |
| sorbinil  | 18.2## 18.3## 24.5## | 12.9## 8.7## 9.25   | 2.3## 1.75## 2.12##  | 256## 4.56## 5.6    |
| LPS       | 58.3 ± 65.8 ± 81.2 ± | 39.6 ± 28.23 ± 25.97 ± | 5.7 ± 6.7 ± 6.55 ±   | 52.7 ± 42.97 ± 35.20 ± |
| sorbinil  | 12.45## 14.2* 5.42*  | 8.4** 2.35* 1.78    | 2.89* 0.7* 1.68*     | 6.9** 2.9* 4.2      |

The Balb/c mice (25 g) were pre-injected i.p. with sorbinil (25 mg/kg body wt/day) for 2 days or carrier followed by single i.p. injection of LPS (4 μg/kg body wt). Sorbinil injections were given at every 24 h after LPS injection. The mice were killed at 1, 3 and 7 days after LPS-injection and liver, heart and spleen tissues were dissected out and (10%) homogenates were prepared in phosphate buffered saline. The cytokines/chemokines levels were measured in the homogenates by using BD biosciences Mouse Inflammation Cytometric bead array kit. All data are expressed as mean ± S.E.M. * P value < 0.05, ** P value < 0.001 as LPS +sorbinil compared to LPS group. # P value < 0.05, ## P value < 0.001 as control group is compared to LPS group.
Fig. 1
Fig. 2

AR activity (mU/μg protein)

A

B

C

GAPDH

Un-transfected

Transfection reagent

Control siRNA

AR siRNA

AR activity

**
Fig. 3

- TNF-α
- IL-6
- IL1-β
- MCP1
- GAPDH

|          | Control | Sorbinil | Tolrestat | Zopolrestat | Control | Sorbinil | Tolrestat | Zopolrestat |
|----------|---------|----------|-----------|-------------|---------|----------|-----------|-------------|
| LPS      |         |          |           |             |         |          |           |             |
FIG. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 9
Fig. 10
