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Inhibition of Aldose Reductase Prevents Endotoxin-Induced Inflammation by Regulating Arachidonic Acid Pathway in Murine Macrophages

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Abstract

Bacterial endotoxin, lipopolysaccharide (LPS) is known to induce release of arachidonic acid (AA) and its metabolic products which play important role in inflammatory process. We have shown earlier that LPS-induced signals in macrophages are mediated by aldose reductase (AR). Here we have investigated the role of AR in LPS-induced release of AA metabolites and their modulation using a potent pharmacological inhibitor fidarestat and AR-siRNA ablation in RAW 264.7 macrophages, and AR-knockout mice peritoneal macrophages and heart tissue. Inhibition or genetic ablation of AR prevented the LPS-induced synthesis and release of AA metabolites such as PGE₂, TXB₂, PGI₂ and LTB₄ in macrophages. LPS-induced activation of cPLA₂ was also prevented by AR inhibition. Similarly, AR inhibition also prevented the calcium ionophore A23187 –induced cPLA₂ and LTB₄ in macrophages. Further, AR inhibition with fidarestat prevented the expression of AA metabolizing enzymes such as COX-2 and LOX-5 in RAW 264.7 cells and AR-knockout mice derived peritoneal macrophages. LPS-induced expression of AA metabolizing enzymes and their catalyzed metabolic products were significantly lower in peritoneal macrophages and heart tissue from AR-knockout mice. LPS-induced activation of redox-sensitive signaling intermediates such as MAPKs, transcription factor NF- κ B as well as Egr-1, a transcription regulator of mPGES-1, which in collaboration with COX-2 leads to the production of PGE₂, were also significantly prevented by AR inhibition. Taken together, our results indicate that AR mediates LPS-induced inflammation by regulating AA metabolic pathway and thus provide novel role of AR inhibition in preventing inflammatory complications such as sepsis.

Keywords

LPS; Inflammation; Aldose reductase; Arachidonic acid; COX-2 and LOX-5

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1. Introduction

Lipopolysaccharide (LPS), a pro-inflammatory endotoxin present in the outer envelope of Gram-negative bacteria, is known to elicit systemic and local host responses [1–3]. LPS is released from the surface of replicating Gram-negative bacteria into the circulation, where it is recognized by a variety of cells including macrophages. The macrophages get activated and trigger induction of pro-inflammatory genes including cytokines, chemokines and other inflammatory markers such as eicosanoids, prostanooids and leukotrienes. These inflammatory markers act in an autocrine or paracrine manner to induce and amplify the host response and defense systems that help eliminate the bacterial infection [2]. However, uncontrolled and excessive bacterial endotoxin in circulation results in the overwhelming amount of inflammatory marker expression in macrophages that induces acute or chronic inflammatory processes including sepsis and endotoxemia. Chronic inflammation also plays a critical role in several cardiovascular and neurological degenerative diseases as well as cancer [4]. Reactive oxygen species (ROS) are key players in these inflammatory processes. Several investigators have studied the mechanism of ROS mediated inflammation in various experimental models; however a clear understanding is still elusive. Hence, elucidation of the mechanism that mediates and regulates inflammatory signals is of profound importance in order to understand a large array of disease processes mentioned above.

Several studies have demonstrated that ROS modulate arachidonic acid (AA) metabolism and formation of eicosanoids in activated macrophages [3] and [5]. In mammalian cells, phospholipase A (PLA2) enzymes have been implicated in catalyzing the cleavage of membrane-bound arachidonate at the sn-2 position thereby releasing AA, an unsaturated fatty acid (20:4, n-6) [2] and [3]. Cyclooxygenase-2 (COX-2) and lipoxygenase-5 (LOX-5) are the critical enzymes implicated in further metabolism of AA. The COX-catalyzed products, prostaglandins (PGs) and thromboxane (TXs) play a major role in inflammatory reactions which are the main characteristic feature of inflammatory symptoms. The LOX-catalyzed products such as leukotrienes (LTBs) are also potent pro-inflammatory mediators in numerous allergic and inflammatory diseases [6]. Thus, inhibition of AA metabolism should be a potential strategy to block the inflammatory cascade. Conventional nonsteroidal anti-inflammatory drugs (NSAIDs) as well as the selective COX-2 or LOX-5 inhibitors, which primarily exert their anti-inflammatory activity either by decreasing the production of prostaglandins (PGs) and/or LTBs, are known to cause adverse cardiovascular thrombotic effects [7]. Recent studies have shown that inhibition of (either COX or LOX) pathway alone could shift the metabolism of AA through the other, resulting in redundancy of the drug, and thus remain ineffective [8]. Therefore, it is argued that the drugs that synergistically block both COX- and LOX-catalyzed metabolic pathways of AA could be more effective as anti-inflammatory agents with a better safety profile than existing NSAIDs, and selective COX-2/LOX-5 inhibitors.

Our recent studies suggest that aldose reductase (AR) is an important mediator of ROS-induced inflammatory signals initiated by cytokines and growth factors (GF) that lead to cytotoxicity in a variety of in-vitro cellular models including macrophages [9–17]. We have also shown that AR inhibition blocks LPS-induced activation of COX-2 and prostaglandin PGE2 (16). These finding suggest that inhibition of AR could modulate AA metabolic cascade, however the detailed mechanism is not clear. Therefore, using murine macrophage cell line (RAW264.7) challenged with LPS without or with AR inhibition or ablation and peritoneal macrophages (PM) derived from LPS challenged AR-deficient (AR^{-/-}) mice, we have investigated the role of AR in AA metabolism and its effect on LPS-induced inflammation. Our results indicate that AR inhibition/deficiency blocks the formation of inflammatory products of AA metabolism such as PGE2, TXB, PGI2 and LTB4 etc, by blocking the phosphorylation and activation of cPLA2 and MAPK etc and expression of

COX-2 and LOX-5. These results suggest an important mediatory role of AR in LPS-induced activation of AA metabolic pathway in murine macrophages.

2. Materials and Methods

2.1 Materials

Dulbecco's modified Eagle's medium with 4 mM l-glutamine and 4.5 g/l glucose, phosphate-buffered saline (PBS), penicillin/streptomycin solution, trypsin, and fetal bovine serum (FBS) were purchased from Invitrogen Gibco (Grand Island, NY). [5, 6, 8, 9, 11, 12, 14, 15-³H (N)] arachidonic acid was purchased from NEN Life Science (Boston, MA). AR inhibitor, fidarestat was a gift from Sanwa Kagaku Kenkyusho Co., Ltd. (Tokyo, Japan). RIPA cell lysis buffer, antibodies against LOX-5, PGI₂ synthase and β -Actin were purchased from Santa Cruz biotechnology (Santa Cruz, CA) USA. Antibodies against cPLA₂ and phospho-cPLA₂ were obtained from Cell Signaling Inc. (Beverly, MA); and antibodies against thromboxane synthase (TXB synthase), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), heme oxygenase -1 (HO-1), Glutamate-cysteine ligase (GCLC) and COX-2 were obtained from Abcam Inc. (Cambridge, MA). Specific ELISA kits for Thromboxane B₂, Leukotriene B₄, cPLA₂, and sPLA₂, glutathione S-transferase (GST) and COX-2 inhibitor Indomethacin and LOX-5 inhibitor REV 5901 were purchased from Cayman chemical Inc (Ann Arbor, MI). Prostaglandin E₂ (PGE₂) assay kit was obtained from Assay Designs, Inc. (Ann Arbor, MI). Mouse HO-1 EIA kit was obtained from Takara Bio inc. Japan. CD11b⁺ peritoneal macrophages isolation kit was obtained from Miltenyi Biotec Inc. (Auburn, CA). Calcium ionophore A23187, lipopolysaccharide (*E. coli*) and the reagents used in Western blot analysis were obtained from Sigma (Sigma Aldrich, St Louise, MO). All other reagents used were of analytical grade.

2.2 Cell Culture and Animals

RAW264.7 macrophages from American Type Culture Collection (Manassas, VA) were grown in DMEM containing 10% FBS, 1% penicillin/streptomycin in a 95% air 5% CO₂-humidified atmosphere at 37°C. In the cellular studies, where required the RAW264.7 cells were incubated with AR inhibitor, fidarestat (final concentration 10 μ M) for at least 12 h before stimulating with LPS for different time periods. The C57BL/6 wild type and AR^{-/-} mice on C57BL/6 background were maintained in a pathogen-free barrier facility at University of Texas Medical Branch, Galveston, TX [16]. The animals were maintained in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and in accordance with the Institute's Guideline of the Animal Care and Use Committee.

2.3 Determination of AA and its metabolites in macrophage culture media

RAW264.7 cells were seeded in 12-well plate at the density of $\sim 0.35 \times 10^6$ cells/well, with and without AR inhibitor (10 μ M) in the complete growth media. After 24 h, the media were removed and replaced with 1 ml of serum-free DMEM containing 0.1 μ Ci/ml ³H-arachidonic acid and incubated for 16 h. The cells were washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin to remove unadsorbed arachidonic acid and stimulated with 1 μ g/ml LPS for 1 h. Subsequently, the cells were incubated with fresh medium containing AR inhibitor (10 μ M) for additional 18 h. The culture medium was collected and centrifuged for 15 min at 10,000 rpm and supernatant was used to measure the radioactivity using a Beckman liquid scintillation counter (Beckman Coulter, Fullerton, CA).

2.4 Isolation and culture of peritoneal macrophages

Mice were injected intraperitoneally with 2 ml of ice-cold 1× Hanks balanced salt solution (without CaCl₂, MgCl₂, MgSO₄, and phenol red; GIBCO) using 26 gauge needle. After injection, peritoneum was gently palpated and peritoneal fluid was aspirated out using a 20 gauge needle. The peritoneal fluid was centrifuged at 1000 rpm for 5 min at 4°C and cell pellets were used for positive selection of CD11b⁺ cells using CD11b microbeads and magnetic MACS LS column according to manufacturer's instruction (Miltenyi Biotec). Eluted CD11b⁺ cells were analyzed for purity by FACS analysis and were >97% pure. The CD11b⁺ peritoneal macrophages were suspended in RPMI 1640 medium containing 5% fetal bovine serum and 1% penicillin/streptomycin and seeded in 24-well plates for overnight to allow the macrophages to adhere to the plates. To the adherent macrophages, 0.1 µCi/ml ³H-arachidonic acid was added and incubated for overnight. The cells were washed twice with phosphate-buffered saline (PBS) containing 0.1% BSA to remove unadsorbed AA, and incubated for 18 h in complete medium. The culture medium was collected and centrifuged for 15 min at 10,000 rpm and supernatant was used to measure the radioactivity using a Beckman Liquid Scintillation Counter (Beckman Coulter, Fullerton, CA). The cells were lysed in RIPA and the whole cell lysates were used for Western blot analysis.

2.5 RNA interference ablation of AR in RAW264.7 macrophages

AR ablation was performed using AR specific siRNA as described earlier [17]. Briefly, approximately 70% confluent RAW264.7 cells in 6-well plates were incubated with serum-free medium containing the AR-siRNA (AATCGGTGTCTCCAACTTCAA) or scrambled siRNA (AAAATCTCCCTAAATCATACA) suspended in DharmaFect transfection reagent (Dharmacon) as suggested by the supplier to a final concentration of 100 nM. After 48 h of incubation, Changes in the expression of AR were determined by Western blot analysis using AR antibodies and by determining enzyme activity in the whole cells lysate.

2.6 Western Blot Analysis

The confluent RAW264.7 cells were incubated with or without AR inhibitor, fidarestat (10 µM), in a 6-well plate for 12 h followed by treatment with 1 µg/ml LPS for another 12 or 24 h. Similarly, AR siRNA or control siRNA-transfected macrophages were serum-starved and incubated with 1 µg/ml LPS for additional 24 h. The cells were washed twice with ice-cold PBS and lysed in ice-cold RIPA lysis buffer. The crude lysate were cleared by centrifugation at 12,000g for 10 min at 4°C. An equal amount of macrophage cell lysate (40 µg) were separated on 10 % SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were incubated in blocking solution containing 5% w/v dried fat-free milk and 0.1% v/v Tween-20 in Tris-buffered saline. Subsequently, the membranes were incubated with specific antibodies against cPLA₂, Phospho-cPLA₂, COX-2, LOX-5, TXB synthase, PGI₂ synthase, Nrf2, HO-1 and GCLC. The blots were then washed, exposed to HRP-conjugated secondary antibodies (1:5,000 dilution) for 1 h, and the antigen-antibody complex was detected by enhanced chemiluminescence (Pierce, Rockford, IL). The membranes were stripped and probed with antibodies against β-Actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal protein loading.

2.7 Determination of PGE₂, 6k-PGF_{1α} (PGI₂), TXB₂ and cPLA₂ levels

The RAW cells (~0.3×10⁶ cells/well in 6-well plates) were growth-arrested in the serum-free medium without or with AR inhibitors (10 µM) for 24 h followed by incubation with 1 µg LPS/ml 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 LPS for

additional 24 h. The medium was collected from each well and cleared by centrifugation (5000 rpm; 5 min). The level of PGE2 was determined using an assay kit according to the manufacturer's instructions (Assay Designs, Inc.). For TXB2 and cPLA2 assays respective ELISA kits were used according to the manufacturer's instructions (Cayman chemical).

2.8 Reverse transcription-PCR

The RAW264.7 cells were pre-incubated without and with fidarestat (10 μ M) for 12 h followed by incubation with 1 μ g/ml LPS for an additional 6 h. Total RNA was isolated by using RNeasy[®] Mini Kit from Qiagen, USA. Equal amount of RNA was used for RT-PCR using Qiagen[®] one step RT-PCR kit. The gene specific primer (purchased from sigma; Sigma Genosys, USA) sequences were; sense COX-2, 5'-GGT TAC AAA AGC TGG GAA GC-3'; anti-sense 5'-GGG GGT TCC GAG TTT ATA CT-3'; sense 5-LOX, 5'-GGC ACC GAC GAC TAC ATC TAC-3'; anti-sense 5'-LOX, 5'-CAA TTT TGC ACG TCC ATC CC-3'; sense TXB synthase, 5'-ATC AGC CAA GCC TGT GAA CT-3'; anti-sense TXB synthase, 5'-CCT CTC TTC TGC TGC TTG CT-3' and GAPDH sense 5'-TCC ACT CAC GGC AAA TTC AAC G-3'; anti-sense 5'-TAG ACT CCA CGA CAT ACT CAG C-3'. The 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; 30 cycles of 94°C 30 s, 62°C 30 s, 72°C 1 min, and then 72°C 5 min for final extension. PCR products were separated by electrophoresis using 1% agarose-1XTAE gels containing 0.5 μ g/ml ethidium-bromide. The band intensities were analyzed densitometrically by using Kodak image station.

2.9 Preparation of mouse heart tissue homogenates

To examine the effect of AR inhibition on LPS-induced expression of cPLA2, COX-2, LOX-5, PGI2 synthase and TXB synthase in vivo, the wild type and AR null mice were injected with LPS (1 mg/kg body wt) intraperitoneally. After 24 h of LPS injection, mice were euthanized, and heart tissues were separated, homogenized in phosphate buffered saline. Equal amount of proteins from homogenates were subjected to SDS-PAGE followed by Western blot analysis as described in section 2.6.

2.10 Determination of total GST activity in LPS-treated macrophages

Macrophages were incubated without or with fidarestat (10 μ M) and LPS (1 μ g/ml) for 24 h. Total GST activity was determined by using a GST assay kit from Cayman Chemicals as suggested by the manufacturer. Briefly, 150 μ l of assay buffer, 20 μ l of glutathione, and 20 μ l of a sample were added in triplicate in 96 well plates. CDNB was used as a substrate and the reaction was monitored by recording absorbance at 340 nm using an ELISA plate reader. The specific activity of GST was expressed as nmol/min/mg protein.

2.11 Statistical analysis

Data presented as mean $\pm$ SE and P-values were determined by unpaired Student's t test using Microsoft Office Excel 2007 software. P < 0.05 was considered as statistically significant.

3. Results

3.1 AR inhibition prevents release of AA metabolites from LPS-induced macrophages

In order to understand the anti-inflammatory efficacy of AR inhibition against bacterial endotoxin, LPS, we first quantified the AA metabolites in macrophages. Macrophages were incubated with ³H-AA followed by stimulation with LPS with or without AR inhibitor. As shown in the Fig. 1, the release of pre-labeled AA and its metabolites increased >4-fold

upon LPS stimulation as compared to un-stimulated RAW264.7 cells. These results indicate that LPS-challenge induced an increased AA metabolites formation and that AR inhibition prevented these changes significantly.

3.2 AR inhibition prevents LPS-induced release sPLA2 and activation of cPLA2 in macrophages

Since in mammalian cells PLA2 is well known to catalyze the release of AA from membrane phospholipids upon stimulation with LPS [18], we next examined effect of AR inhibition on the activation of cellular PLA2 (cPLA2) as well as release of soluble PLA2 (sPLA2) in LPS-treated macrophages. As shown in Fig. 2A the levels of sPLA2 significantly (>2 fold) increased after LPS challenge and inhibition of AR by fidarestat significantly (>80%) prevented this increase. Similar results were observed in AR-siRNA transfected macrophages treated with LPS (Fig. S1). Further, approximately two-fold increase in the cPLA2 activity upon LPS-challenge was also prevented by AR inhibition in macrophages (Fig. 2B). These results suggest that AR regulates the PLA2 activity during inflammatory conditions.

3.3 AR inhibition/ablation prevents LPS-induced production of AA pathway- generated lipid mediators in macrophages

Since AA pathway-generated lipid mediators such as prostaglandins (PGs), thromboxane (TXs) and leukotrienes (LTs) play a major role in inflammatory complications [19], [20] and [21], we next examined whether AR inhibition/ablation could affect LPS-induced generation of PGE2, TXB2, 6k-PGF1 α and LTB4 in macrophage culture media. As shown in Fig. 3A there was more than 2.5 fold increase in PGE2 in LPS-treated cells as compared to control cells and AR inhibition significantly decreased levels of PGE2 in the medium upon LPS-challenge. Further, there was approximately 29-fold increase in TXB2 levels in LPS-challenged RAW264.7 culture medium and whereas in AR inhibitor TXB2 levels were significantly (95%) decreased after LPS challenge (Fig. 3B). Since, PGI2 has a very short half-life and readily converted to 6k-PGF1 α , we determined the levels of 6k-PGF1 α in RAW264.7 cells. Upon stimulated with LPS, a significant (approximately 4-fold) increase in the levels of 6k-PGF1 α was observed which was significantly prevented by AR inhibition (Fig. 3C). We next measured the effect of AR inhibition on the LPS-induced release of LTB4, a reaction catalyzed by LOX-5, in macrophages. As shown in Fig. 3D, LPS-stimulated RAW264.7 cells released approximately 2-fold more LTB4 in the medium than control cells. Further, inhibition AR with fidarestat significantly prevented LPS induced increase in LTB4. Similar results were observed in the cells transfected with AR-siRNA followed by treatment with LPS (Fig. S2). These results suggest that AR regulates the release of inflammatory lipid mediators upon LPS stimulation in RAW264.7 cells. Since calcium ionophore A23187 is also well known to rapidly increase the production of lipid mediators such as LOX and cPLA2, we examined if AR inhibition could prevent A23187 – induced increase in the macrophage cPLA2 and LTB4. Macrophages were therefore incubated with calcium ionophore A23187 (1 μ M) for 60 min without or with fidarestat (10 μ M). Our results shown in the Fig. 4A and B indicate that the levels of cPLA2 and LTB4 were significantly increased to ~4 fold and ~2 fold, respectively in the macrophages treated with A23187 and fidarestat prevented this increase by >65%. These results suggest that AR inhibition could also prevent production of lipid mediators independent of transcriptional regulation of their respective genes.

3.4 AR inhibition prevents LPS-induced activation of AA metabolic enzymes in macrophages

Since synthesis of various AA metabolic products such as PGE2, TXB2, PGI2, and LTB4 is catalyzed by enzymes such as COX-2, TXB synthase, PGI2 synthase and LOX-5,

respectively, we next investigated the effect of AR inhibition on LPS-induced expression of these proteins in macrophages. As shown in Fig 5A and 5B, LPS challenge resulted in approximately 10-fold increase in COX-2 expression and 8.5 fold increase in TXB synthase expression. These changes were significantly (~70 %) prevented by AR inhibition. Similarly, approximately 4-fold increase in PGI2 synthase and LOX-5 protein expressions were observed upon LPS challenge in macrophages, which were significantly prevented by AR inhibitor fidarestat and the values were similar to controls (Fig. 5C and 5D).

Further, we have also measured the effect of AR inhibition on LPS-induced mRNA levels of these enzymes. As shown in Fig. 6A–C, LPS challenge resulted in approximately 4-fold increase in the expressions of COX-2, and LOX-5 and 8-fold increase in TXB synthase expression. However, macrophages pretreated with AR inhibitor followed by LPS challenged showed significantly (~95%) decreased expression of COX-2, TXB synthase, and LOX-5 genes. These results indicate that AR regulates AA metabolic enzymes at the level of transcription.

3.5 AR null mice are resistant to LPS-induced generation of AA pathway-generated lipid pro-inflammatory mediators

In order to validate our in-vitro results with macrophage cell line RAW264.7, we used peritoneal macrophages (PM) isolated from AR null mice to investigate whether AR deficiency could modulate LPS-induced AA metabolites. At first, we examined the release of AA metabolites in the culture medium of PM derived from LPS-challenged wild type and AR^{-/-} mice. Our results shown in the Fig. 7A indicate that LPS caused a significant 4-fold increase in AA metabolites in the PM isolated from wild-type mice, where as PM isolated from AR^{-/-} mice showed only 2-fold increase. Further, we determined the LPS-induced expression of various enzymes involved in AA metabolism in PM isolated from WT and AR^{-/-} mice. There was 4 to 8-fold increase in the expressions of cPLA2, LOX-5, COX-2, PGI2 synthase and TXB synthase enzymes upon stimulation with LPS in WT PM where as in AR^{-/-} derived PM the expression of these enzymes were not affected upon LPS challenge and remained similar to unstimulated control values (Fig. 7B).

We next measured the levels of various AA metabolites such as PGE2, 6k-PGF1 α , TXB2 and LTB4 in PM derived from WT and AR^{-/-} mice and found that LPS stimulation caused more than 4-fold increase in the levels of PGE2, 6-fold in the levels of 6k-PGF1 α , and ~3.5-fold in the levels of TXB2 and LTB4 in culture medium of PM isolated from WT mice. The increases in the levels of various lipid mediators were significantly low in AR^{-/-} mice treated with LPS (Fig. 8A–D). Since bacterial endotoxin-induced cardiomyopathy is one of the major contributing factor in mortality associated with sepsis and related inflammatory complications, we next determined the expression of various AA metabolizing enzymes in WT and AR null mice heart tissues. Our results shown in the Fig 9A to 9F, indicate that the expressions of cPLA2, COX-2, LOX-5, PGI2 synthase and TXB synthase were significantly (from 2-fold to 6-fold) elevated in the heart tissue obtained from LPS-challenged WT mice. However, no such increase in the expression of cPLA2, COX-2, LOX-5, PGI2 synthase and TXB synthase were observed in the hearts of LPS-treated AR^{-/-} mice.

3.6 AR inhibitor prevents LPS-induced macrophage death and monocyte adhesion to endothelial cells better than specific inhibitors of COX-2 or LOX-5

Since our results indicate that inhibition of AR affects both COX-2 and LOX-5 pathways, next we compared the effects of AR inhibition with specific inhibitors of COX-2 and LOX-5, respectively. As shown in Fig. 10A, LPS-induced decrease in macrophage viability was significantly prevented by AR inhibition whereas COX-2 inhibition by indomethacin or LOX-5 inhibition by REV 5901 was not significantly effective in preventing cell death.

Further, we tested effects of these inhibitors on the monocyte adhesion to vascular endothelial cells in-vitro and found that LPS caused an approximately 60% increase in monocyte adhesion as compared to controls without LPS-stimulation. Though inhibition with COX-2 and LOX-5 inhibitors prevented LPS-induced monocyte adhesion to HUVEC, AR inhibitor was significantly more effective in preventing the adhesion (Fig. 10B). These results suggest that AR inhibitor, fidarestat is most potently inhibits the adhesion of monocytes to endothelial cells as well as prevention of LPS-induced macrophages death as compared to specific COX and LOX inhibitors.

3.7 AR inhibition regulates LPS-induced activation of NF- κ B and Nrf-2 signals

Subsequent to determining the role of AR in mediating LPS-induced alteration in AA metabolism, we next investigated the probable molecular mechanism by which AR inhibition prevents formation of AA metabolites in macrophages. Since expression of COX-2 and other AA metabolizing enzymes are known to be regulated by MAPK-NF- κ B pathway, we examined LPS-induced phosphorylation and activation of p38, ERK 1/2 and p65 subunit of transcription factor NF- κ B. Our results shown in the Fig 11, indicate that LPS challenge caused a time-dependent increase in phosphorylation of p38, ERK 1/2 and p65 in macrophages. The phosphorylation of these kinases and activation of NF- κ B were significantly prevented in macrophages treated with fidarestat prior to LPS challenge. Since Egr-1 is a known regulator of microsomal PGES-1 (mPGES-1), we next determined whether AR inhibition could prevent LPS-induced phosphorylation of Egr-1. As shown in Fig. 11, LPS caused a time-dependent increase in the phosphorylation of Egr-1 and incubation of macrophages with fidarestat prior to LPS-stimulation significantly prevented the phosphorylation. Thus our result suggests that LPS-induced alteration of AA metabolism could be regulated by AR via modulating the activation of MAPK, NF- κ B and Egr-1 signaling intermediates. Since Nrf-2 is known to provide protection against oxidative stress –induced inflammation by inducing anti-inflammatory proteins such as HO-1, GST and GCLC, we next examined the effect of AR inhibition on LPS-induced activation of Nrf-2 and its dependent expression of anti-inflammatory proteins in macrophages. LPS caused a time-dependent increase in the nuclear levels of Nrf-2 and AR inhibitor significantly exacerbated it (Fig. 12A). Incubation of macrophages with AR inhibitor alone also increased the Nrf-2 levels though much less than that induced by LPS. Further, as shown in the Fig. 12B and 12C, LPS-caused increased expression of HO-1 and GCLC, and the activation of total GST which was further exacerbated by AR inhibition. Thus our results provide a novel mechanism that AR inhibition could prevent LPS-induced inflammatory response by preventing NF- κ B – dependent signals and activating the Nrf-2-dependent signals.

4. Discussion

LPS, a Gram-negative bacterial cell wall component with high pro-inflammatory capabilities, is known to induce various cytotoxic and inflammatory changes via activation of phospholipases (PLA) and AA metabolism. Several studies have shown that exposure of different cells such as monocytes [22], leukocytes [23] and vascular smooth muscle cells [24] to bacterial endotoxin LPS leads to activation of PLA2, which releases AA from membrane lipids subsequent to LPS stimulation. Further, COX and LOX enzymes metabolize AA into PGE2, TXB and LTB4, major inflammatory lipid mediators. The products of AA metabolism are implicated in various inflammatory diseases such as sepsis [25] and endotoxemia. Therefore, regulation of AA metabolism by inhibiting or decreasing the expression of key AA metabolic enzymes such as cPLA2, COX-2 and LOX-5 could be an effective mechanism for the prevention of inflammation and related pathologies. A number of natural products such as resveratrol and curcumin have been shown to inhibit COX-2 expression and are well known anti-inflammatory agents [26], [27], [28] and [29].

Curcumin has been shown to alter the AA metabolism by blocking the phosphorylation of cPLA2 and decreasing the expression of COX-2 and LOX-5 [29]. Recently, we have demonstrated that benfotiamine, a lipid-soluble analogue of vitamin B1 inhibits cPLA2 activation, COX-2 expression and PGE2 release in murine macrophages [30]. It appears that inhibition of cPLA2 phosphorylation and subsequent regulation of AA release and metabolism could be an effective mechanism for controlling inflammation.

In the present study, we observed that LPS-induced activation of phospholipases, production of AA and formation of AA metabolites which could contribute to the LPS-induced inflammation in macrophages were significantly prevented by AR inhibition or ablation. COX-2-metabolized products are essential for our body's defense against infections as they alert the immune cells against invading pathogen. However, overproduction of COX-2 metabolites results in acute and chronic inflammatory disorders. Therefore, Non-steroidal Anti-inflammatory Drugs (NSAIDs) are used for the treatment of various inflammatory conditions by targeting COX-2; however, side effects of NSAIDs such as gastric ulcerations or cardiovascular dysfunctions due to nonselective inhibition of COX-1 and COX-2 are major concerns in their therapeutic use [31], [32] and [33]. Hence, specific inhibitors that specifically target the enzymes that synthesize eicosanoids and cause inflammation with minimal side effects are required urgently for effective intervention in inflammatory pathogenesis. We have shown earlier that AR inhibition significantly prevents LPS-induced activation of COX-2, PGE2 synthesis and subsequent inflammation [34]. In the present study, we have demonstrated that inhibition of AR by a very potent and specific inhibitor, fidarestat could prevent LPS-induced inflammation by preventing the activation of cPLA2 and aberrant AA metabolism in macrophages. Further, we have demonstrated that inhibition of AR effectively inhibits the release of AA metabolites in murine macrophages. We validated these results using PM isolated from LPS-treated WT and AR^{-/-} mice and observed that the release of AA metabolites was significantly decreased in AR^{-/-} mice derived PM as compared to WT mice derived PM. These results suggest that AR deficiency protects mice from LPS-induced inflammation by blocking the release of AA metabolites.

LPS-induced increase in the COX activity is believed to be mainly due to inducible COX; i.e., COX-2, which has been shown to be dependent upon activation of cPLA2 via a feedback mechanism as cPLA2 provides the substrate (AA) for COX-2 [35]. AA is further metabolized by COX-2 into prostaglandin G2 (PGG2), which upon conversion to prostaglandin H2 (PGH2) serves as the substrate for specific prostaglandin synthases such as PGI2 synthase and TXB synthase [36]. In the present study, we investigated whether the expression of various synthases is affected by AR inhibition. Our results demonstrate that AR inhibition with fidarestat significantly decreased the expression of enzymes that synthesize PGE2 and TXB2 (fig. 3A–D). Thus, inhibition of COX-2 in combination with down-stream PG synthases appears to be responsible for the decreased biosynthesis of pro-inflammatory PGs. It is however, likely that decreased expression of PG synthase enzymes and subsequent decreased biosynthesis of pro-inflammatory prostaglandins could be due to the feedback mechanism and secondary to the lesser amount of substrate's availability after COX-2 inhibition by AR inhibitor. PGI2 is another major product of the AA metabolism formed by the action of PGI2 synthase [37]. Increased expression of PGI2 synthase and its catalytic product PGI2 has been reported in patients under septic shock and in animals treated with LPS or pro-inflammatory cytokines [38], [39] and [40]. We also observed that LPS-challenge lead to increased expression of PGI2 synthase in murine macrophages which was significantly prevented upon pretreatment with AR inhibitor. Once formed, PGI2 gets converted into 6-ketoPGF1 α , a crucial player in vasodilation resulting in reduced blood pressure, one of the symptoms during sepsis [38]. In the present study, AR inhibitor significantly prevented the increase in 6-ketoPGF1 α levels, which could be due to, and subsequent to, the decreased levels of PGI2 upon AR inhibitor mediated decrease in the

COX-2 expression and its catalytic product, PGH₂, which is a substrate for PGI₂. These results suggest that AR inhibition could be effective in bacterial infections- induced vasculopathy involving PGI₂.

A number of studies have demonstrated that leukotrienes are important regulators of cancer cell proliferation and cause apoptosis in non-cancer cells [41], [42], [43], [44], [45] and [46]. Since, AA is metabolized via LOX-5 into leukotrienes, specific inhibition of LOX-5 may shunt the AA metabolism by LOX-5 leading to the decreased formation of leukotrienes. Therefore, we examined whether AR inhibition by fidarestat could regulate the expression of LOX-5. Our results demonstrate that LPS-induced expression of LOX-5 in RAW264.7 cells was significantly prevented by fidarestat (Fig. 5D). These results thus indicate that AR inhibition prevents the activation and/or expression of two key regulatory enzymes of AA metabolism i.e. COX-2 and LOX-5, thereby regulates the synthesis of inflammatory mediators such as eicosanoids, prostanoids and leukotrienes. These results strongly suggest that potent and highly specific inhibitors of AR such as fidarestat could be more effective in preventing inflammatory complications as opposed to broad and nonspecific anti-inflammatory drugs such as NSAIDs or individual inhibitors of either COX-2 or LOX-5 enzymes which leaves them redundant. Indeed, our results shown in Fig. 10 indicate that AR inhibitor, fidarestat most potently inhibits LPS-induced adhesion of monocytes to endothelial cells as well as cell death in macrophages as compared to specific COX-2 and LOX-5 inhibitors, suggesting that inhibition of AR could be advantageous over the individual inhibitors of either COX-2 or LOX-5 in preventing LPS induced pathological changes.

Although a clear mechanism of an anti-inflammatory role of AR inhibition is still awaited, our previous evidences and observations indicate that AR inhibition prevents activation of PLC/PKC/MAPK/NF- κ B pathway in murine macrophages [12] and [47]. Several investigators have shown that LPS-induced activation of cPLA₂ in cell membrane is directly or indirectly mediated via protein kinase C (PKC) and mitogen-activated protein kinase (MAPKs) [48–54]. Further, studies have demonstrated that expression of COX-2 and mPGES-1 is regulated by transcription factors NF- κ B and Egr-1, respectively; therefore, we investigated if AR inhibition could affect activation of these transcription factors. Our results from the current study demonstrate that AR inhibition prevented LPS-induced increase in phosphorylation of NF- κ B and Egr-1 along with that of MAPK-p38, ERK 1/2, in macrophages. Our recent studies also indicate that AR-catalyzed- reaction product, glutathionyl-1,4-dihydroxynonene (GS-DHN) mediate oxidative stress signals in macrophages as well as smooth muscle cells by activating NF- κ B via PKC (12, 55). These studies thus suggest that AR-catalyzed lipid peroxidation products mediate the formation of LPS-induced inflammatory mediators. However, the precise mechanism(s) by which these aldehydes mediate LPS-induced inflammatory signals still need to be investigated. Further, our current studies also indicate that AR inhibitors up-regulate the expression of anti-inflammatory proteins such as HO-1 and GST via Nrf-2 –dependent mechanism. Thus our studies provide a novel role of AR inhibitors in regulating both Nrf-2 –dependent anti-inflammatory and NF- κ B-dependent- pro-inflammatory pathways induced by LPS in macrophages. In summary, by inhibiting LPS-induced phosphorylation and activation of the signaling intermediates, AR inhibition could block the expression of key regulatory enzymes of AA metabolism such as cPLA₂, COX-2 and LOX-5 and subsequent biosynthesis of pro-inflammatory metabolites which ameliorate inflammation in murine macrophages. Further, dual regulation of COX and LOX pathways by AR inhibition could be a novel mechanism by which AR inhibition exhibits potential anti-inflammatory response in endotoxemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AR	Aldose reductase
AA	Arachidonic acid
LPS	Lipopolysaccharide
PGE2	Prostaglandin E2
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PGI2	Prostaglandin synthase
TXB	Thromboxane
LTB4	Leukotrienes
cPLA2	Cytosolic phospholipase A2
sPLA2	soluble phospholipase A2
COX-2	Cyclooxygenase-2
LOX 5	Lipoxygenase-5
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase
NF-kB	Nuclear Factor-KappaB
Egr-1	Early growth response factor 1
ROS	Reactive oxygen species
NSAID	Nonsteroidal anti-inflammatory drugs
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
HO-1	Heme oxygenase-1
GCLC	Glutamate-cysteine ligase – catalytic
GST	Glutathione S-Transferase

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Highlights

- Aldose reductase (AR) regulates the formation of arachidonic acid metabolites.
- AR inhibition prevents LPS-induced PGs, TXBs and LTBs.
- AR inhibition prevents LPS-induced expression of cPLA2, sPLA2, Cox-2 and Lox-5.
- AR inhibition prevents NF- κ B and activates Nrf-2 in macrophages.
- AR inhibitors could be novel anti-inflammatory drugs in the prevention of sepsis.

Figure 1.

AR inhibition prevents the release of arachidonic acid (AA) metabolites from LPS-stimulated RAW264.7 cells. The RAW254.7 cells were incubated with ³H-AA (0.1 μ Ci/ml) as described in methods and stimulated with 1 μ g/ml LPS for 1 h. The cells were washed and incubated for 18 h in fresh medium containing AR inhibitor (10 μ M). The release of metabolites in the medium was determined by measuring the radioactivity in the culture medium. Data represents mean $\pm$ S.E. ($n=4$). # $p < 0.001$ as compared with AA alone, ** $p < 0.001$ as compared with AA+LPS-treated cells; AA; Arachidonic acid, Fida; Fidarestat

Figure 2.

AR inhibition prevents LPS-induced production of sPLA₂ and cPLA₂ in RAW264.7 macrophages. (A & B) The RAW cells were growth-arrested in medium containing 0.1% serum without or with AR inhibitor (10 μ M) as indicated and challenged with LPS for 18 h. The sPLA₂ levels in the culture medium of RAW cells were determined by using the monoclonal enzyme immunoassay kit as described in methods. (B) The cPLA₂ levels in cell homogenate were determined by using the monoclonal enzyme immunoassay kit as described in methods. Data represents mean $\pm$ S.E. ($n=4$). # $p<0.001$ Vs control; ** $p<0.001$ Vs LPS treated cells; \$ $p<0.01$ Vs control cells; ## $p<0.01$ Vs. LPS-treated cells.

Figure 3.

Effect of AR inhibition on LPS-induced production of PGE₂, TXB₂, 6k-PGF_{1α} and LTB₄ in RAW264.7 macrophages. The RAW cells were growth-arrested in Dulbecco's modified Eagle's medium containing 0.1% serum with or without AR inhibitor (10 μM) and challenged with LPS for 18 h. (A-D) The levels of PGE₂, TXB₂, 6k-PGF_{1α} and LTB₄ were determined in the culture medium by using the monoclonal enzyme immunoassay kits as described in methods. Data represents mean ± S.E. (n=4). [#] *p* < 0.001 Vs control cells; ^{**} *p* < 0.001 Vs LPS-treated cells; ^{\$} *p* < 0.05 Vs control cells; ^{##} *p* < 0.05 Vs LPS-treated cells. C; Control, Fida; Fidaresstat, L; LPS, Sc; Scrambled SiRNA, siAR; AR siRNA, AR; Aldose reductase.

Figure 4.

Effect of AR inhibition on calcium ionophore A23187-induced release of cPLA2 and LTB4 in RAW264.7 macrophages. The RAW cells were growth-arrested overnight in medium containing 0.1% serum without or with AR inhibitor (10 μ M). Subsequently macrophages were challenged with 1 μ M calcium ionophore A23187 for 1 h. The levels of cPLA2 and LTB4 were measured in the cell homogenates and culture media by using monoclonal enzyme immunoassay kits. Data represents mean $\pm$ S.E. ($n=4$). * $p<0.001$ Vs control; ** $p<0.001$ Vs A23187-treated cells; C; Control, Fida; Fidarestat.

Figure 5.

AR inhibition prevents LPS-induced expression of COX2 and TXB synthase, PGI2 synthase and LOX-5 in RAW cells. The RAW cells were growth-arrested by incubating in 0.1% FBS medium without or with AR inhibitor, fidarestat (10 μ M), and stimulated with LPS (1 μ g/ml) for 24 h. Cytosolic extracts were prepared and equal amounts of cytosolic proteins were subjected to Western blot analysis using antibodies against (A) COX-2, (B) TXB synthase (C) PGI2 synthase (D) LOX-5 and (E) β -Actin. A representative blot from each group is shown. Data represents mean $\pm$ S.E. ($n=3$). # $p < 0.001$ Vs control; ** $p < 0.001$ Vs LPS treated cells. Cont; Control and Fida; Fidarestat.

Figure 6. Effect of AR inhibition on LPS-induced COX2 and TXB synthase, and LOX-5 gene mRNA expression in RAW cells. Cells were growth-arrested in Dulbecco's modified Eagle's medium containing 0.1% serum with or without the indicated AR inhibitors (10 μ M) and challenged with LPS (1 μ g/ml). The total RNA was isolated and reverse transcriptase-PCR analysis was carried out using specific primers for COX-2, TXB synthase and LOX-5. Equal amount of PCR products were electrophoresed with 1% agarose-TAE gels containing ethidium bromide. Reverse transcriptase-PCR analysis with GAPDH served as control. Data represents mean $\pm$ S.E. ($n=3$). # $p<0.001$ Vs control; ** $p<0.001$ Vs LPS treated cells. Cont; Control and Fida; Fidarestat.

Figure 7.

Peritoneal macrophages isolated from AR null mice are resistant to LPS-stimulated release of AA metabolites. (A) Peritoneal macrophages isolated from mice peritoneum were incubated with (0.1 $\mu\text{Ci}/\text{ml}$) ^3H -AA with/or without LPS for 12 h at 37°C. The release of AA metabolites was determined by measuring the radioactivity in the culture medium. (B) The peritoneal macrophages were lysed in RIPA lysis buffer and equal amount of proteins were subjected to Western blot analysis using antibodies against (a) cPLA2, (b) LOX-5, (c) COX-2, (d) Thromboxane synthase, (e) PGI2 synthase and (f) GAPDH. Representative blots are shown ($n = 3$). Data represents mean $\pm$ S.E. ($n=3$). [#] $p < 0.001$ Vs control wild type cells; ^{**} $p < 0.0001$ Vs LPS treated wild type cells. C-wt; control wild type, C-ARKO; control-

aldose reductase knockout, L-wt; LPS treated wild type, L-ARKO; LPS treated-aldose reductase knockout.

Figure 8.

Peritoneal macrophages isolated from AR null mice are resistant to LPS-stimulated production of inflammatory lipid mediators. LPS-induced peritoneal macrophages isolated from AR knockout mice peritoneum were growth-arrested in RPMI-1640 containing 0.1% serum for 24h. The levels of PGE2, 6k-PGF1 α , TXB2, and LTB4 were determined in the culture media using the monoclonal enzyme immunoassay kits as described in methods. Data represents mean $\pm$ S.E. ($n=4$). [#] $p < 0.001$ Vs control wild type cells; ^{**} $p < 0.001$ Vs LPS treated wild type cells. C-wt; control wild type, C-ARKO; control-aldose reductase knockout, L-wt; LPS treated wild type, L-ARKO; LPS treated-aldose reductase knockout.

Figure 9.

Decreased expression of AA pathway enzymes in the hearts of LPS-treated AR null mice. The ARKO and WT mice were injected (i.p) with LPS and after 24 h heart tissues were dissected out. Equal amount of proteins from mice heart homogenates were analyzed by Western blotting using antibodies against (A) cPLA2, (B) LOX-5, (C) COX-2, (D) Thromboxane synthase, (E) PGI2 synthase and (F) GAPDH. Representative blots are shown. Data represents mean $\pm$ S.E. ($n=3$). [#] $p < 0.001$ Vs control; ^{**} $p < 0.001$ Vs LPS treated cells. C-wt; control wild type, C-ARKO; control-aldose reductase knockout, L-wt; LPS treated wild type, L-ARKO; LPS treated-aldose reductase knockout.

Figure 10.

Effect of inhibitors of AR, COX-2 and LOX-5 on LPS-induced macrophage viability and monocyte adhesion to endothelial cells. (A) Growth-arrested macrophages were preincubated in the presence or absence of AR inhibitor fidarestat (10 μM), COX-2 inhibitor indomethacin (10 μM) and LOX-5 inhibitor REV 5901 (10 μM) for 24 h at 37°C. Cell viability was determined by trypan blue exclusion method. (B) Serum-starved HUVECs cells were preincubated without or with fidarestat, indomethacin and REV 5901 for 24 h, followed by stimulation with LPS (1 $\mu\text{g}/\text{ml}$) for another 24 h. After incubations, the cells were washed with PBS, U-937 cells were added, and the incubation continued for another 12 h. Cell adhesion was determined by MTT assay. Data represents mean $\pm$ S.E. (n=4). [#]p<0.001 Vs control; ^{**}p<0.001 Vs LPS-treated cells.

Figure 11.

Effect of AR inhibition LPS-induced p38, ERK1/2 and p65 phosphorylation in macrophages. Growth-arrested macrophages were treated with LPS without and with fidarestat (10 μ M) for indicated time at 37°C. Cell lysate was prepared and equal amount of protein was subjected to western blot analysis using antibodies against A. phospho p38 and p38 B. phospho pERK1/2 and ERK1/2, C.EGR-1, D. phospho p65 and p65 and E. GAPDH. Data represents mean $\pm$ S.E. ($n=3$). # $p < 0.001$ Vs control; ** $p < 0.001$ Vs LPS treated cells.

Figure 12.

AR inhibition up-regulates LPS-induced expression of Nrf2, HO-1 and GCLC, and activity of GST in macrophages. Macrophages were growth-arrested by incubation in 0.1% FBS medium without or with AR inhibitor, fidarestat (10 μ M) for 12 h followed by stimulation with LPS (1 μ g/ml). (A) At indicated time points, nuclear and whole cell extracts were prepared. Nuclear proteins were separated by SDS-PAGE, and western blot analysis was conducted using Nrf2 antibodies. (B) At 12 and 24 h, equal amounts of whole cell extracts were subjected to Western blot analysis using antibodies against HO-1 and GCLC and the blots were stripped and reprobbed with loading control GAPDH. (C) At 24 h, total GST activity was measured using CDNB as substrate as described in methods. Data represents mean $\pm$ S.E. ($n=3$). * $p<0.01$ and ** $p<0.001$ Vs untreated cells.