ALDOSTERONE INDUCES IL-8 PRODUCTION IN HUMAN MONONUCLEAR MACROPHAGE THROUGH MINERALOCORTICOID RECEPTOR AND TOLL-LIKE RECEPTOR-4

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ABSTRACT

Stress and inflammation contribute to increased morbidity and mortality associated with activation of the rennin-angiotensin-aldosterone system (RAAS), learnt from our previous trauma researches. Because aldosterone has been implicated in the stimulation of cytokines production in various tissues, we investigated whether it would affect IL-8 production in cultures of human mononuclear macrophage. We first demonstrated that treatment with 10 to 10^4 nM aldosterone leads to a significant increase in IL-8 secretion and mRNA levels in a time-dependent manner. Pretreatment of cells with the mineralocorticoid receptor (MR) antagonist spironolactone and anti-Toll-like receptor 4 (anti-TLR4) antibody inhibited IL-8 production in aldosterone-treated cultures. We also demonstrated that aldosterone increases IL-8 production by time dependent manner. We further discovered that aldosterone could regulate the protein expression of MR and TLR4. Thus, we have demonstrated for the first time that aldosterone, which stimulates IL-8 production through the MR-dependent pathway, also maybe a novel endogenous ligand to TLR4.

Keywords*: mineralocorticoid receptor, aldosterone, TLR4, genomic.*

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Introduction

Aldosterone, a major member of the rennin-angiotensin-aldosterone system (RAAS), is recognized as a major regulator of ionic homeostasis by modulating cation transport. Beyond its renal effects on sodium reabsorption, aldosterone excess elevated concentrations can exert deleterious effects on the endothelial dysfunction, inflammation and oxidative stress[(1)]. Aldosterone acts through the mineralocorticoid receptor (MR) to regulate the expression of inflammation genes or affect signal transduction via genomic and nongenomic pathways involving transactivation of epidermal growth factor receptor and activation of extra-cellular signal-regulated kinase and other kinases and pathway[(2)].

Moreover, seminal studies in humans and rat models suggest that endogenous aldosterone increases inflammatory biomarkers through an MR-dependent or MR-independent pathways[(3)].

Aldosterone increased aortic chemotactic protein-1(MCP-1) expression and caused monocyte/macrophage infiltration in the adventitia and perivascular fat. The cross talk between aldosterone-stimulated MR and angiotensin type 1a receptors(AGTR1a) plays a role in vascular effects of aldosterone[(4)]. In stress-dependent hypertensive rat strain, increased aldosterone level was observed, indicating aldosterone as a stress-induced hormonal responsible for the stress-dependent and then for sustained blood pressure elevation[(5)]. The adrenocortical cells produce various inflammatory

cytokines such as TNF-a and IL-6, and in Japanese aldosterone-producing adenomas patients, the mRNA expression levels of TNF-a, and NFKB1 were significantly greater and IL-6 tended to be greater[6]. These results implicated that activation of the RAAS are required to inflammation.

Toll-like receptors (TLRs) are an increasingly conserved family of pattern recognition receptors central to the innate immune response to infection, and have an important role in innate immune responses and subsequent activation of acquired immune responses[7]. TLRs can recogonize a variety of microbial pathogens and the initial induction of immune and inflammatory responses. Emerging studies described that innate immune activation through TLRs is an important driver in the pathogenesis of vascular remodelling and endothelial dysfunction, and renal injury[8-9].

Recent evidence also suggests that TLRs can-recognize endogenous ligands, including the high mobility group box 1 protein(HMGB1), mitochondrial DNA and heparin sulfate[7-9], which known as damage-associated molecular patterns (DAMPs). TLRs expressed by cells of the innate immune system and these cells almost exist in the vascular system. Additionally, Wistar rats infused with aldosterone and 1% NaCl for four weeks augmented the cardiac and renal expression of TLR4. They links this augment with higher expression of cytokines(TNF-α, IL-1β and MCP-1), and cardiac and renal fibrosis because a TLR4 signaling inhibitor, TAK-242, reversed these alterations.The uses of TLRs antagonism not only demonstrated their pivotal role in the fibrotic process, but also its participation in the inflammation[10]. The similar proinflammatory effect implicated the relationship between the TLRs and aldosteron. The demonstration of aldosterone as a endogenous ligand and a inflammatory mediator deserves further research.

Our present study focused on the effects of aldosterone and its antagonist on the production of proinflammatory cytokine (IL-8) in cultures of human monocytes. The present study was designed to extend these findings, determine the dose-response relationship, and elucidate the time course and mechanism of this effect. These studies preferred the human monocyte cell line, THP-1, which produces the normal range of human cytokines. We raise the hypothesis that aldosterone stimulates IL-8 production through the MR and TLR4-dependent pathway, also maybe an endogenous ligand to TLR4.

Materials and methods

Materials and reagents

Cell culture media (RPMI 1640), L-glutamine, penicillin/streptomycin, and phosphate-buffered saline (PBS) solutions were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Biochrom (Berlin, Germany). Polymyxin B, Triton X-114, LPS (endotoxin, Escherichia coli 0111:B4), aldosterone, and spironolactone were purchased from Sigma Chemicals (St. Louis, MO). Mouse antihuman TLR4 (HTA125, cat no. ab30667) monoclonal antibody were from Abcam (Hong Kong, China), and mouse antihuman mineralocorticoid receptor antibodies (cat no.MAB4369) were from R&D Systems, Inc., (Minneapolis, MN).

Cell culture

The human monocyte THP-1 cell line was purchased from the Shanghai Type Culture Collection (China), with catalog TCHu 57. Cells were grown in RPMI 1640 media (Invritrogen, Carlsbad, USA) supplemented with L-glutamine, 10% fetal bovine serum (Biochrom, Germany), penicillin/streptomycin (1000 units/ml and 50 μg/ml, respectively), 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate, and 2.25 g of glucose. The cells were cultured in 75-cm2 tissue culture flasks (Corning, USA) at 37°C in a 5% CO2 atmosphere. The cells were maintained in logarithmic growth (2×10^5 to 1×10^6/ml) by passage every 3 to 4 days. The cell viability was detected in every cell preparation by trypan blue exclusion and found to be greater than 95%. The final cell concentration was 10^6 cells per ml. 12 h prior to stimulation cells were grown in medium without any supplements and antibiotics. For stimulation cells were incubated in supplement-free medium together with aldosterone (SigmaChemicals, Deisenhofen, Germany) ($10\sim10^4$nM) for different times, and IL-8 released in the conditional medium was measured.

IL-8 ELISA

The cells (10^6/ml per well) were incubated with or without stimulant in RPMI 1640 medium without fetal bovine serum for 30 min to 12 h in 24-well round-bottomed plates. Cells were spun down at 600×gfor 5 min and the supernatants were harvested and subjected to the IL-8 ELISA kit (R&D Systems, Minneapolis, USA). Add 300μl of stan-

dard diluentto6tubes labeled as 1000, 500, 250, 125, 62.5, 31.3, 15.6 pg/ml. 100μl of homogenized samples and standards were dispensed into the appropriate wells, and then 100μl of biotinylated anti IL-8 was mixed into each well. The mixture was covered and incubated for 60min at room temperature. Following three washes with 250μl wash buffer, 100μl of the avidin-HRP solution was added to each well. The plate was then covered and incubated for 30min at room temperature. After additional washes, 90μl of the TMB was added and incubated for 20-25min in the dark at room temperature. Within 30min of adding the stop solution, the absorbance at 450nm was measured, and the results were calculated using a four-parameter curve fit. The results were expressed as pg/ml.

Western Blot

Cell proteins were extracted using Cell Lysis Buffer (Abcam) according to the manufacturer's instructions. Protein concentration was measured with Bradford Protein Assay Reagent (Fementas, Burlington, CA) and separated in polyacrylamide gels and electro-transferred onto PVDF membranes. After blocking the membrane at room temperature for 6 h, the membrane was incubated for 1.5 h with various primary antibodies specific for TLR4(HTA125, cat no. ab30667)(Abcam, Hong Kong, China) and MR(cat no.MAB4369) (R&D Systems, Minneapolis, MN) respectively. After incubation with HRP-conjugated secondary antibodies for 1h at 25°C, the signals were visualized by EnlightTM Western Blotting Reagents (Engreen Biosystem, China) according to the manufacturer's instruction.

Confocal Microscopy

At the end of the 12 h incubation period with the indicated treatment, THP-1 cells were fixed in 3.7% paraformaldehyde-phosphate-buffered saline at 37°C for 10 min and permeabilized in 0.2% Triton X-100 for 10 min. After cells were washed in PBS, the slides were blocked with 0.5% normal goat serum at 37°C for 1 h. The cells were stained with primary antibodies to the MR for 1 h at 37°C and then a FITC-labeled secondary anti-mouse Fab2 was added for 1h at 37°C. Nuclei were counterstained with propidium iodide. All of the cultures were then mounted in Elvanol and examined with a Zeiss microscope attached to a cooled charge-coupled device camera and a computer-generated video analysis system (Image-Pro Plus software).

Immunocytochemical analysis

THP-1 cells were fixed in the mixture of methanol and acetone with 1:1 volume for 30 min at room temperature and smeared on glass coverslips. Coverslips were saturated with 0.02% polylysine and processed for anti-TLR4 antibody, followed by biotin labeling Ig and diaminobenzidin. Between all incubation steps, cells were washed three times for 5 min with PBS containing 0.2% BSA. Coverslips were mounted on slides and images were analyzed using a microscope (Nikon, Japan).

Quantitative RT-PCR

Total RNA samples were isolated from THP-1 cells using the E.Z.N.A. Total RNA Kit (Hoffmann-LaRoche, Nutley, US), and digested with RNase-free DNase I (Fermentas) following the manufacturer's instructions. Real-time quantitative RT-PCR analyses were performed with the ABI Prism 7,300 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green I PCR reagents (Fermentas). To determine exact copy numbers of the target genes, quantified concentrations of subcloned PCR fragments of IL-8, TLR4, and MR were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of total RNA samples were used for each real-time RT-PCR. Samples were measured in duplicate and expression was calculated relative to the housekeeping gene β-actin using $\triangle$CT method. Primers sequences used were shown in table 1.

Target		Sequence	
Actin	Sense	TGCGCAGAAAACAAGATGAG	114bp
	Antisense	CACCTTCACCGTTCCAGTTT	
IL-8	Sense	TCTGCAGCTCTGTGTGAAGG	100bp
	Antisense	AAATTTGGGGTGGAAAGGTT	
TLR4	Sense	TCATTGTCCTGCAGAAGGTG	143bp
	Antisense	CAGGGCTTTTCTGAGTCGTC	
MR	Sense	CAAGTCGTGAAGTGGGCAAAGGTA	121bp
	Antisense	ACGATCTCCAGCTCAAGGCAAATG	

Tab. 1: Primers used for the amplification.

LPS content in ALD

Contaminating LPS in protein preparations was removed by phase separation using Triton X-114 as previously described. Briefly, 1/20 volume of Triton X-114 was added to ALD solution. After 10 min of gentle rotation at room temperature, the

samples were centrifuged for 5 min (6,000 ×g) at room temperature, and the top layer (containing ALD) was carefully aspirated and saved. The LPS content in ALD preparations was measured by the Gram Negative Endotoxin Determination Reagents according to the manufacturer's instructions (Gold Mountainriver Tech, BeiJing, China). Afer the treatment, LPS content in ALD dilution is typically lower than 0.3832EU/ml.

Immuno precipitation

THP-1 cells were stimulated with 10nM of ALD, 10nM of ALD-BSA (Usbio), control IgGand vehicle control (BSA) for 12 h and extracted in immune precipitation assay buffer containing protease inhibitors. Cells were placed on ice, scraped, and transferred to a 1.5-ml tube. Cells were sonicated for 20 min on ice and then spun down at 1387×g for 5 min at 4 °C. Cell lysate was transferred to a clean tube and stored at -20 °C. Protein concentration was determined by Bradford Protein Assay (Fementas) according to the manufacturer's instructions. 500μg of total protein was pre-cleared with 1μg of control IgG antibody was incubated overnight at 4 °C and 20 μl of Protein A/G-Plus agarose (Millipore) for 30 min at 4 °C while rotating. Beads were pelleted by centrifuging at 1387×g for 30 s at 4 °C, and lysate was removed and placed in a new tube.

To the pre-cleared cell lysate, 2 μg of rat anti-mouse TLR4 primary antibody (Abcam) was added, and the mixture was incubated overnight at 4 °C. 20μl of Protein A/G-Plus agarose was added, and the mixture was incubated at 4 °C rotating for 3 h. Beads were pelleted by centrifuging at 1387×g for 30 s at 4 °C. Beads were washed five times with ice-cold PBS, spinning down as described after each wash. After the last wash, the supernatant was removed and beads were resuspended in 40μl of sample buffer for Western blotting.

Statistical Analysis

Results are expressed as the means±SD of triplicate independent experiments. Differences were statistically evaluated by one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison test for multiple groups at a significance level of $P<0.05$.

Results

Aldosterone Up-Regulates IL-8 Production and Gene Expression in an Time- Dependent Manner in Cultures of THP-1

We first demonstrated that treatment of cultured human monocytes with 10 to 10^4 nM aldosterone leads to a significant increase in the steady-state level of IL-8 production in the supernatants and 10 nM aldosterone also up-regulates the effective expression of IL-8 gene in a time-depend manner (Figure 1A and B).

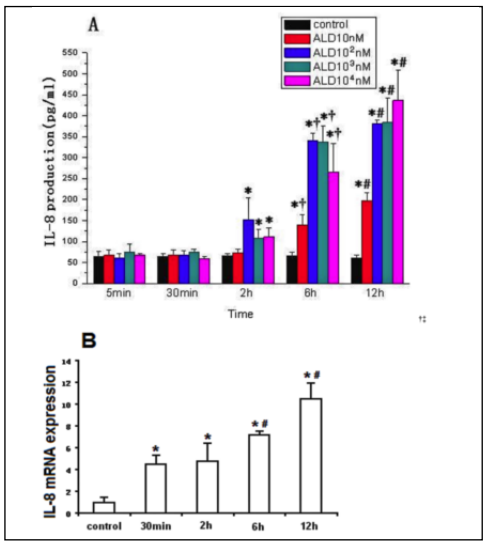

Figure 1: The effect of aldosterone on IL-8 production and mRNA expression in cultures of human monocytes THP-1. **A**: ELISA analysis was used to assess IL-8 production in cultures treated from 5 min to 12 h with 10-104nM aldosterone. The results demonstrate that aldosterone time-dependently increased IL-8 levels compared with values of a shorter time stimulation (* $P<0.05$ vs. 5 min group, $^{\dagger}P<0.05$ vs. 5 h group, $^{\#}P<0.05$ vs. 6 h group) **B**: Realtime PCR analysis was used to assess IL-8 mRNA transcripts in cultures treated from 30 min to 12 h with 10nM aldosterone. The results indicate that aldosterone time-dependently increased IL-8 mRNA expression from 2 h to 12 h compared with untreated control values ($^{*\#}P<0.05$).

The basal values of IL-8 production were 64.814±5.574 pg/ml, and then we found that a significantly increase of IL-8 production after 2 h stimulation of ALD under different dosage. The production increasingly raised by the time passed.

Under different dosage, the production was higher than the production under a shorter time of stimulation. A short time of stimulation (5 min to 30 min) could not lead to the up-regulation of IL-8 production.

Therefore, we think that ALD induced IL-8 production in a time-dependent manner from 2 h to 12 h in THP-1 cells and exclude the possibility of rapid nongenomic effects of aldosterone. However, we didn't found a dose-dependent manner of ALD to IL-8 production, because the higher dosage of ALD didn't lead to a higher production of IL-8. The overmuch of aldosterone may be thought interactions with the glucocorticoid receptor(GR), which may explain the slight inhibitory effect after 2 h or 6h of stimulation.

We next evaluated whether ALD-stimulated increases in IL-8 production led to corresponding increases in mRNA expression (Figure 1B). On the basis of our data and data from several published studies that used concentrations for in vitro experiments, we selected 10nM aldosterone for further study[14-15]. We cultured the THP-1 cells with ALD (10nM) for 30 min, 2 h, 6 h, and 12 h. We prepared RNA, converted the RNA to cDNA, and performed the PCR reaction. The mRNA expression of IL-8 increased 4.7, 7.2 and 10.5-fold above baseline upon exposure of THP-1 cells to 10nM ALD after 2 h, 6 h, and 12 h. Thus, we confirmed that ALD stimulates THP-1 cells to secrete IL-8 in a time-dependent manner.

Aldosterone Up-Regulates IL-8 Production and regulates IL-8 Gene Expression via MR and TLR in Cultures of THP-1

To investigate whether or not the observed aldosterone effects occurred via the MR or TLR, we pretreated monocytes with 1μM spironolactone and 10μg/μl anti-TLR4 monoclonal antibody for 30 min and then co-incubate with ALD for 12h to observe the blocked effect.

Figure 2 indicates that pretreatment of human monocytes with spironolactone, which abolished ALD-induced increase in IL-8 production measured by an ELISA assay(Fig. 2 A). Both spironolactone and TLR4 antibody prevent an aldosterone-induced increase in IL-8 mRNA expression measured by the Real-time PCR assay(Fig. 2 B). The observations suggest that ALD probably induces IL-8 through the MR and TLR4-dependent process. Combination with the time course results, we think the up-regulation of ALD was typically classic genomic action.

ALD-stimulated monocytes IL-8 secretion is LPS independent

LPS is a potent activator of monocytes activities. Indeed, it is known that LPS induced cytokines production in a dose-dependent manner from 1 to 100 ng/ml in THP-1[16-17]. To establish that ALD-induced IL-8 secretion could not be explained by LPS contamination, we supplemented cultures with polymyxin B, a well-characterized pharmacologic LPS antagonist. Although polymyxin B had no effect on IL-8 secretion by ALD (380.439±9.878 vs 384.820±77.652 pg/ml), it clearly suppressed LPS-stimulated IL-8 secretion from 2708.595±48.631 to 1312.887±125.639 pg/ml (Fig. 3).

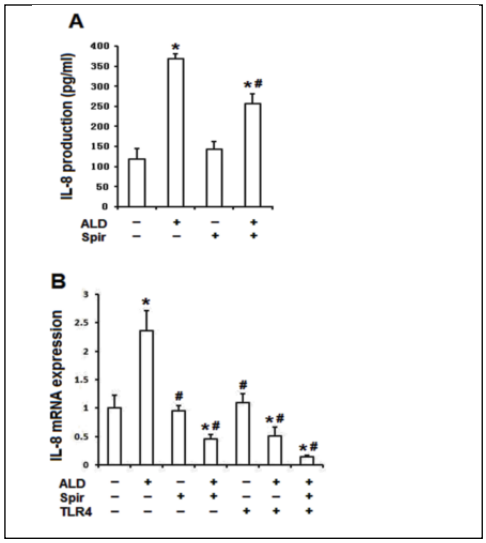

Figure 2: The effect of MR antagonist-spironolactone and TLR4 monoclonal antibody on IL-8 production and mRNA expression in cultures of human monocytes THP-1. A: MR antagonist-spironolactone inhibited ALD-induced increase in IL-8 production in THP-1 cultures. ELISA analysis was used to assess IL-8 production in cultures treated 12 h with 10nM ALD (*P<0.05 vs. the control, #P<0.05 vs. ALD group). B: Spironolactone and TLR4 monoclonal antibody abrogate ALD-induced increase in IL-8 gene expression. Real time PCR analysis was used to assess IL-8 gene expression treated 12 h with 10nM ALD. The inhibitor and neutralizing antibody were preincubated with monocytes for 30 min before ALD was added. *Statistically different from control group and #from ALD group (P<0.05).

This result indicated sufficiently that ALD-stimulated IL-8 secretion is LPS independent. Although published studies routinely use 10μg/ml polymyxin B to antagonize LPS, polymyxin B did not significantly affect ALD-induced IL-8 secretion even at 25 μg/ml; an higher concentrations was toxic to monocyte cultures.

Aldosterone Regulates MR Protein Expression in Cultures of THP-1

To further examine whether MR would be involved in the initiation of the cellular signaling

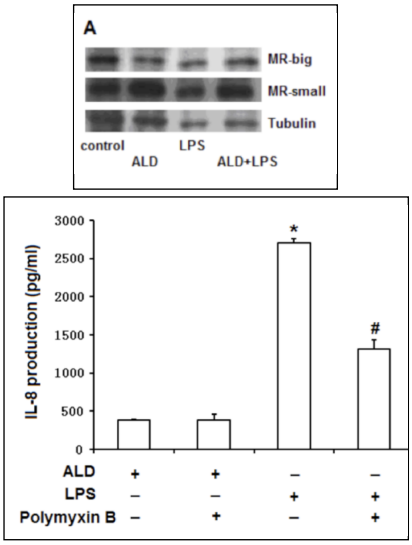

Figure 3: ALD-stimulated IL-8 secretion is not LPS dependent. THP-1 cells were stimulated with the indicated quantities of ALD (10nM) or LPS(10ng/ml) in the presence or the absence of the pharmacologic LPS antagonist polymyxin B sulfate (25 μg/ml). After 12 h supernatants were harvested and assayed for IL-8 by ELISA. *Statistically different from ALD group and #from LPS group ($P<0.05$).

leading to an ALD-induced increase in IL-8 production, the MR protein expression was measured after 12 h stimulation of ALD (10nM) and LPS (10ng/ml) by western blot (Figure 4).

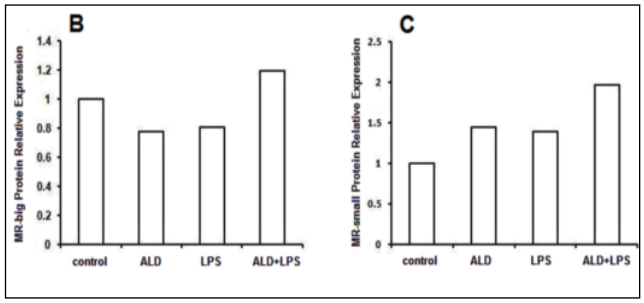

Figure 4: Western blot of MR in cultures of THP-1 after 12 h stimulation of ALD (10nM) and LPS (10ng/ml). Relative optical densities of MR are expressed as the band density of each group.

Tubulin corrected the experimental error as a housekeeping control. The western blot demonstrated that there are two protein straps, the big one (~110 kDa) and the small one (~50 kDa), respectively. Our results indicated that the protein of big strap decreased 22.7% and 19.7%, while the small strap increased 44.2% and 39.2% in ALD and LPS group, respectively. We though the receptor was modified or dissociated with chaperones then translocated into nucleus for transcription and translation.

Aldosterone Up-Regulates TLR4 Protein Expression in Cultures of THP-1

We further examined the expression of TLR4 at protein levels.

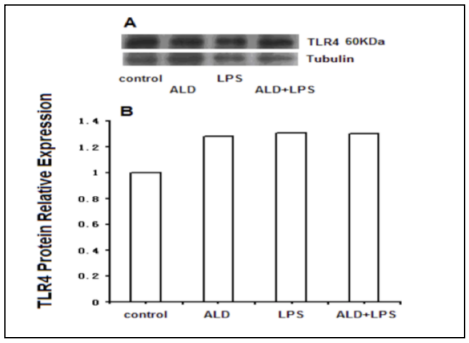

Figure 5: Western blot of TLR4 in cultures of THP-1 after 12 h stimulation of ALD (10nM) and LPS (10ng/ml). Relative optical densities of TLR4 are expressed as the band density of each group.

In Figure 5, ~60 kD bands corresponding to TLR4 were observed by Western blotting of total cell lysates from THP-1 cells treated with ALD (10nM) and LPS (10ng/ml) for 24 h. Western blot analysis revealed that the cell surface expression of TLR4 was also strongly upregulated 1.28-fold and 1.30-fold in ALD and LPS-treated cells when compared with the control cells. These results indicate that treatment of THP-1 cells with ALD strongly enhanced TLR4 expression at protein level.

Immunofluorescence and immunocytochemistry analysis of association of ALD with MR and TLR4

To investigate the association of ALD with MR and TLR4, we measured molecular interactions of ALD with each of these receptors by immunofluorescenceand immunocytochemistry, using these images to reveal protein-protein interactions after ALD stimulation of THP-1 cells.

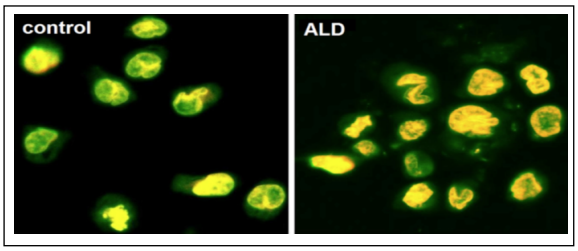

Figure 6 A: Immunofluorescence analysis of association of ALD with MR in cultures of THP-1. Cells were incubated with ALD (10nM) for 12 h and then labeled with anti-MR. The yellow color for merged staining identifies the nucleus. The fluorescent signals generated by MR molecules were shown in the cytoplasm and nucleus. Two additional experiments generated similar results.

As shown in Figure 6A, at the initial time point, the fluorescent signals generated by MR molecules were low-density, and almost appeared in the cytoplasm, whereas after 12 h stimulation of ALD, the signals reinforced and translocated into and nucleus partly. The images suggested that the MR were activated by 10nM of ALD. As shown in Figure 6B, the TLR4 was lowly expressed on the plasma membrane at the control group, but after 12 h stimulation of ALD, the density was obviously increased, indicated an activated receptor.

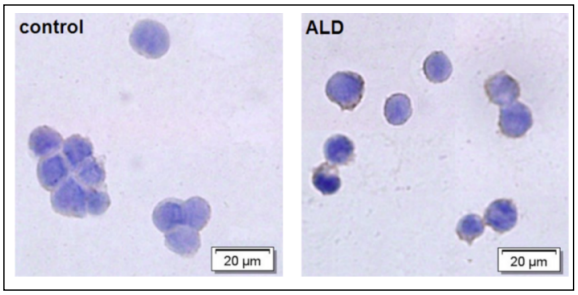

Figure 6 B: Immunocytochemistry analysis of association of ALD with TLR4 in cultures of THP-1. Cells were incubated with ALD (10nM) for 12 h and then labeled with anti-TLR4. TLR4 was expressed on the plasma membrane. Two additional experiments generated similar results.

Immunoprecipitation analysis of association of ALD with TLR4

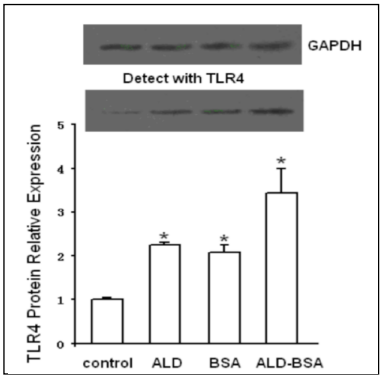

Figure 7: THP-1 cells were stimulated with 10nM of ALD, 10nM ofALD-BSA, and vehicle control (BSA) for 12 h, and protein was extracted using immune precipitation assay buffer plus protease inhibitors. Immunoprecipitations were carried out with anti-ALD antibody, and Western blots were probed with anti-TLR4 antibody. Graphs depict the mean ± SD of data from three individual experiments.*Statistically different from control group ($P<0.05$).

Following the release of IL-8 initiated from ALD, we realized that maybe a previously unrecognized cooperation between TLR4 and ALD is important to inflammation in stress and injury. The monocytes were stimulated with control IgG, vehicle control, ALD, or ALD-BSA for 12 h at room temperature, then cells were washed and subjected. Because of the low molecular weight, we investigated the ALD-BSA to reduce the difficulty of western bloting, and chose BSA as the vehicle control, additionally. Immunoprecipitation of protein in cell extracts with an antibody against ALD was capable of co-precipitating TLR4 (Figure 7). Thus, we believe that TLR4 physically associates with and functionally recognizes ALD.

Discussion

In this study we demonstrate that:

1) the human monocytes possess the functional MR and TLR4, secrete IL-8 and express IL-8 mRNA;

2) IL-8 secretion by THP-1 is increased by aldosterone;

3) aldosterone stimulate IL-8 in an MR and TLR4 dependent manner and that blocking the receptors coincides with IL-8 production. To the best of our knowledge, this is the first report of the modulation of IL-8 secretion in the monocytes by aldosterone. These results suggest that aldosterone plays a central role in the stress and inflammation, connecting the RAAS system and the immune system.

Aldosterone has been shown to promote pathological inflammation via MR activation[18], which the inflammation plays central roles in the pathogenesis of diseases[19], such as atherosclerosis, immune dysfunction and so on. ALD stimulates the activation of proinflammatory transcription factors nuclear factor-kappar B (NF-κB), which induces the production of adhesion molecules, chemokines such as monocyte chemoattractant protein (MCP)-1, and inflammatory cytokines. Results of clinical and animal trials that showed that the MR antagonist spironolactone and eplerenone exerted cardioprotective effects were primarily connected to the mechanisms that prevent vascular inflammation and collagenous fibrosis[20-22].

Fiebeler et al. investigated the contribution of MR activation to proinflammatory and profibrotic mediators in the heart of rats doubly transgenic for the human renin and angiotensinogen genes. MR antagonism prevented vascular injury and activation of inflammation mediator, such as activator protein-1 and NF-κB. Rocha et al.[23] demonstrated that 4-week treatment with ALD and salt caused extensive inflammatory arterial lesions with

perivascular macrophages in the heart. MR blockade decreased this inflammatory response. ALD also increased the expression of intercellular adhesion molecule, cyclooxygenase-2, and MCP-1, effects that were decreased by MR blockade. These studies are in agreement with our results about the pro-inflammatory effect of ALD.

It has also been shown that MR antagonists do not abolish all ALD-induced effects. Early studies suggest that there is unknown membrane receptor distinct from the classical cytoplasmic MR, which involved in the genomic and nongenomic effects of ALD[24]. Although full structural characterization of this putative receptor (or receptors) has not been completed yet[25], data suggest that some MR-independent effects of ALD occur after activation of the pertussis toxin-sensitive heterotrimeric G proteins[26-27].

The existence of MR-independent action lead to the further researches. Yide Zhang et al.[28] found that cardiac and renal expression of TLR4 is increased by ALD infusion, which results in an activation of inflammatory response. A TLR4 inhibitor, TAK-242 reverses these alterations. Here, we demonstrated that although the MR antagonist abolished the effect of ALD, it did not eliminate the up-regulation effect of this hormone. As shown in Figure 2B, blocking the TLR4 with a specific monoclonal antibody (10μg/ml), eliminated the stimulatory effect of ALD on IL-8 mRNA expression. We reported that TLR4 is involved in ALD complex effect for the first time and the stimulatory effect of ALD on IL-8 is mediated via MR and TLR4 activation.

TLR4 is best known at present for its ability to recognize LPS on Gram-negative bacteria. Currently, a major limitation in the interpretation of these reports is conclusive evidence that the response observed was not due to undetected contamination by LPS. As LPS is a widely present and active at extremely low concentrations, rigorous demonstration of the identity of a TLR4 stimulatory molecule is required. In the present work, endotoxin determination assays confirmed that all reagents were well below the threshold endotoxin level required for TLR4 activation.

Recently, several investigations have suggested that there are many different endogenous ligands for TLR4. Moreover, more and more ligands will be found in the nearly future. In our experiment, inhibition by specific blocking antibodies, suggests that the increase in human IL-8 induced by ALD is dependent on TLR4.The data indicated that TLR4 maybe a novel receptor can recognize the signal expressed by ALD and ALD maybe an endogenous ligand for TLR4.

The present experiments indicate that both MR and TLR4 are involved in IL-8 up-regulation by ALD. Although the participation of multiple receptors in ALD signaling is somewhat surprising, similar results have been reported by other investigators, who also found that ALD could activate cells through MR-dependent and -independent pathways[28]. Such findings demonstrate that ALD has the potential to interact with more than one receptor, and have been proved by our studies. Future experiments are necessary to identify the specific sequences in ALD that associate with the receptors.

Interaction of ALD with MR and TLR4 results in up-regulation and activation of these receptors, would be expected to result in additional proinflammatory responses, including cytokine release, that contribute to organ dysfunction and death. The finding that ALD associates with both MR and TLR4 indicates that interventions directed against either of these receptors alone would not be able to prevent the inflammatory injury induced by ALD. Understanding the nature of molecular signaling by ALD may prove therapeutically beneficial in the treatment of human stress diseases where excessive amounts of ALD are produced.

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Abbreviations: RAAS, rennin-angiotensin-aldosterone system; MR, mineralocorticoid receptor; TLR4, Toll-like receptor 4; TLRs, Toll-like receptors; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; MCP, monocyte chemoattractant protein; NF-κB, nuclear factor-kappar B.

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