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Lipopolysaccharide Directly Stimulates Aldosterone Production Via Toll-Like Receptor 2 and Toll-Like Receptor 4 Related PI_3K/Akt Pathway in Rat Adrenal Zona Glomerulosa Cells

Hsin-Lei Huang,[1] Ming-Fu Chiang,[2] Chia-Wen Lin,[1] and Hsiao-Fung Pu[1*]

[1]*Department and Institute of Physiology, School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC*

[2]*Department of Neurosurgery, Mackay Memorial Hospital, Graduate Institute of Injury Prevention and Control, Taipei Medical University, Taipei, Taiwan, ROC*

ABSTRACT

The level of circulating endotoxin is related to the severity of cardiovascular disease. One of the indexes for the prognosis of cardiovascular disease is the plasma aldosterone level. Recently, the Toll-like receptors (TLRs), lipopolysaccharide (LPS)-regulated receptors, were found not only to mediate the inflammatory response but also to be important in the adrenal stress response. Whether LPS via TLRs induced aldosterone production in adrenal zona glomerulosa (ZG) cells was not clear. Our results suggest that LPS-induced aldosterone secretion in a time- and dose-dependent manner and via TLR2 and TLR4 signaling pathway. Administration of LPS can enhance steroidogenesis enzyme expression such as scavenger receptor-B1 (SR-B1), steroidogenic acute regulatory protein (StAR) and P450 side chain cleavage (P450scc) enzyme. LPS-induced SR-B1 and StAR protein expression are abolished by TLR2 blocker. Furthermore, we demonstrated that phosphorylation of Akt was elevated by LPS treatment and reduced by TLR2 blockers, TLR4 blockers, and LY294002 (PI_3K inhibitor). Those inhibitors of PI_3K/Akt pathways also abolish LPS-induced aldosterone secretion and SR-B1 protein level. In conclusion, LPS-induced aldosterone production and SR-B1 proteins expression are through the TLR2 and TLR4 related PI_3K/Akt pathways in adrenal ZG cells. J. Cell. Biochem. 111: 872–880, 2010.

KEY WORDS: LPS; ALDOSTERONE; TOLL-LIKE RECEPTOR; Akt; SR-B1; StAR

Chronic mild endotoxemia is common in several illnesses, such as dialysis patients, and it is related to the severity of cardiovascular disease [Dijk et al., 2001; Szeto et al., 2008]. Long-term inflammation can lead to intravascular coagulation, endothelial dysfunction, and cardiovascular disease. It is noteworthy that aldosterone plays an important role in those pathological change [Weber, 2001]. Aldosterone, secreted by adrenal zona glomerulosa (ZG) cells, regulates the fluid/electrolyte balance and blood pressure. In ZG cells, steroidogenesis-related proteins, whose functions include cholesterol uptake and the activity of synthetic enzyme, decide the rate of aldosterone production [Rocha et al., 1999; Stier et al., 2002; Rossi et al., 2005]. However, angiotensin II (Ang II) type 1 receptor (AT_1R) blockade decreases lipopolysaccharide (LPS)-induced inflammation and aldosterone production in the adrenal gland [Sanchez-Lemus et al., 2008]. Moreover, it has been shown that LPS exerts different effects in adrenocorticotropic hormone (ACTH)- and Ang II-induced aldosterone secretion by ZG cells, although the effect on basal secretion was not determined [Enriquez

Abbreviations used: TLRs, Toll-like receptors; LPS, lipopolysaccharide; ZG, zona glomerulosa; SR-B1, scavenger receptor-B1; StAR, steroidogenic acute regulatory protein; P450scc, P450 side chain cleavage enzyme; Ang II, angiotensin II; AT_1R, Ang II type 1 receptor; PI_3K, phosphoinositide 3-kinase; DOC, deoxycorticosterone; RIA, radioimmunoassay.

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Ming-Fu Chiang and Hsiao-Fung Pu contributed equally to this work.

The authors declare no conflicts of interest.

*Correspondence to: Dr. Hsiao-Fung Pu, PhD, Department and Institute of Physiology, School of Medicine, National Yang-Ming University, Shih-Pai, Taipei, Taiwan 11221, ROC. E-mail: hfpu@ym.edu.tw

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de Salamanca and Garcia, 2003]. Therefore, there are no studies describing that LPS acts directly on adrenal ZG cells to stimulate aldosterone production.

One of the recognition systems to induce innate immune responses is Toll-like receptors (TLRs), which require many molecules including CD14, MD-2, and LPS-binding protein to recognize and respond to invading microorganisms [Palsson et al., 2004]. TLRs have been found to not only mediate inflammatory response to certain microbial infections but also positively correlate with other human diseases such as cancer and cardiovascular disease [Berg et al., 2009; Machida et al., 2009]. A high rate of TLR2 and TLR4 polymorphisms are also found to positively correlate with human diseases [Bornstein et al., 2006]. LPS, a component of the walls of Gram-negative bacteria, promotes systemic inflammation through specific TLR4 [Hajjar et al., 2001; Kiechl et al., 2002]. Besides, both TLR2 and TLR4 are involved in immune-adrenal crosstalk during LPS administration. Therefore, we used LPS to activate both TLR2 and TLR4 to evaluate adrenal ZG cells function.

In the immune system, the downstream gene expression by TLRs, such as nuclear factor kappa B (NF-κB), is the typical signaling during LPS treatment. LPS also initially promotes a wide spectrum of inflammation responses by regulating numerous signaling mediators including reactive oxygen species and phosphoinositide 3-kinase (PI_3K) [Wang et al., 2000; Liu et al., 2002; Li and Engelhardt, 2006; Kim et al., 2008a]. Among others, PI_3K plays a decisive role in steroidogenesis to regulate steroid hormone production [Meroni et al., 2002].

Previous studies focused only on the adrenal inflammatory response and the hormone release induced by LPS, and they failed to elucidate whether LPS stimulates adrenal cells indirectly via the activation of tissue-resident inflammatory mediators or directly via adrenal TLRs. Moreover, the role of TLRs in the aldosterone production in adrenal gland is still unclear. Therefore, we preformed a comprehensive analysis of aldosterone production after being activated by bacterial lipid, LPS. We also investigated the mechanisms of endocrine response. Our results demonstrated that LPS could act adrenal ZG cells via TLR2 and TLR4 related PI_3K/Akt pathway to stimulate aldosterone production.

MATERIALS AND METHODS

ANIMALS

Male Sprague–Dawley rats (8 weeks old) were purchased from the National Yang-Ming University Animal Center. They were housed under controlled conditions of temperature (22 ± 1°C) and light (14–10 h light-dark) with free access to food and water. All animal experiments were conducted humanely and in compliance with the policy statement of the Committee of National Yang-Ming University.

CELL CULTURE

The method for the preparation of dispersed ZG cells was adopted from the method previously described in detail [Campbell et al., 2003]. Rats were sacrificed and adrenal glands were promptly excised. After removal of excess fat, the capsule fractions (mainly ZG) were incubated with collagenase (5 mg/ml Krebs-Ringer bicarbonate buffer with 3.6 mmol K^+/L, 11.1 mmol glucose/L, 0.2% bovine serum albumin, 100 U/ml penicillin G, and 100 μg/ml streptomycin; pH 7.4) at 37°C for 30 min. The viability was assessed by using the trypan blue exclusion test, and it was about 90–95%. Inner cell contamination in glomerulosa cell preparation was less than 5%. ZG cells were seeded in 24-well plates (5×10^4 cells/well) or 6 cm petri dishes (1×10^5 cells/dish) in culture medium (25 mM HEPES, 3.7 g/L $NaHCO_3$, 2% fetal bovine serum, 10% bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin). Cultures were maintained in a humidified 5% CO_2-95% air atmosphere at 37°C for 3 days.

ALDOSTERONE RADIOIMMUNOASSAY (RIA)

The concentration of aldosterone in the culture media was determined by RIA as described elsewhere [Chang et al., 2008]. The media samples were incubated with aldosterone antiserum (1:3,600 diluted with buffer solution) and 0.1 ml ^{3}H-aldosterone (approximately 8,000 cpm; Amersham, UK) at room temperature for 1 h and then incubated at 4°C for 24 h. An adequate amount of 0.05% dextrane T70-coated charcoal was added and the samples were further incubated in an ice bath for 30 min. After centrifugation (200*g*, 4°C for 10 min), the supernatant was mixed with 3 ml liquid scintillation fluid (Ecoscint A, National Diagnostic, Atlanta, GA), and the radioactivity was measured in an automatic β counter (Packard). The sensitivity of the aldosterone RIA was 4 pg/tube. The intra- and inter-assay coefficients of variation were 7.5% ($n = 5$) and 8.1% ($n = 5$) respectively.

WESTERN BLOT ANALYSIS

ZG cells were cultured in 6 cm petri dishes (1×10^6 cells/dish) for 3 days. After drug treatment, cells were lysed via incubation with 50 μl homogenization buffer (1.5% Na-lauroyl-sacrosine, 2.5 mM Tris base, 1 mM EDTA, 0.1% phenylmethyl sulfonylfluoride, pH 7.8), and total protein concentrations were determined by the Bradford protein assay [Zor and Selinger, 1996]. The proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gels and were transferred electrophoretically onto polyvinylidene difluoride membranes. Membranes were blocked with 5% BSA in Tris-buffered saline-0.1% Tween-20 (TBST; 20 mM Tris, 50 mM NaCl, pH 7.6) for 1 h at room temperature and then incubated overnight at 4°C with antibodies against phospho-Akt, Akt, SR-B1, P450scc, StAR protein, or β-actin. After three-time washes with TBST, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rat secondary antibody for 1 h at room temperature. Then, the membranes were visualized with a chemiluminescent detection system (ECL™ Western blotting detection reagents, Amersham International plc., Bucks, UK) as described by the manufacturer.

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ZG cells were cultured on cover glasses in a 24-well plate (5×10^4 cells/well). After drug treatment, cells were fixed with 4% paraformaldehyde in PBS buffer for 20 min and permeabilized with 0.25% Triton X-100 for 15 min at room temperature. After being blocked with 5% goat-serum in PBS, cells were incubated with anti-phospho Akt antibodies at 4°C overnight. Both Rhodamine red-conjugated goat anti-rabbit IgG and DAPI (4,6-diamidino-2-

phenylindile, to visualize nuclei, dilution 1:1,000), were added for 2 h at 37°C. After three-time washes, cover glasses with ZG cells were mounted with Vectashield mounting medium and images were obtained by using a confocal microscope (MRC 1000). Images were taken by using a 100× objective. Data were collected in three different dispersions. To better quantify the translocation occurring in ZG cells, nuclei showing signs of translocation were counted in 12 fields for each condition and plotted against the total number of cells present in each field.

BLOCKING ASSAYS FOR TLR2/TLR4

TLR2 and TLR4 play a role in the response of myeloid cells to LPS or microbial lipoprotein/lipopeptidies. Antibodies to TLR2 and TLR4 have been reported to inhibit the LPS- or lipoprotein/lipopeptidies-induced cytokine by human PBMCs and mouse peritoneal macrophages. Anti-mouse TLR2 (T2.5) or TLR4/MD2 (MTS510) has slightly modified the blocking experiments and they were published with this blocking assay [Akashi et al., 2000; Meng et al., 2004].

ZG cells (1×10^6 cells) were seeded in 6 cm petri dishes. After 24 h of incubation, cells were pre-incubated with anti-TLR4/MD-2 or anti-TLR2 antibody (20 μg/ml) (e-Bioscience) for 1 h at 37°C. Then, 10 μg/ml LPS was added for 10 min, 15 min, or 12 h at 37°C, in a 5% CO_2-95% air humidified incubator. ZG cells were collected and protein expression was analyzed by Western blot.

STATISTICAL ANALYSIS

All data were expressed as mean ± SEM. Data were processed by one-way analysis of variance (ANOVA) by using SPSS system. Multiple comparisons were performed when one-way ANOVA was significant by using the Duncan's and Dunnett's multiple-range test. Differences between means were considered significant when $P < 0.05$.

RESULTS

LPS-INDUCED ALDOSTERONE RELEASE BY RAT ADRENAL ZG CELLS VIA TLR2 AND TLR4

Adrenal ZG cells were incubated with different doses of LPS (0, 0.001, 0.01, 0.1, 1, 10, 100 μg/ml) for 12 h, and aldosterone release increased in a dose-dependent manner (Fig. 1A). Administration of LPS (10 μg/ml) for 6, 12, 24, or 48 h, aldosterone release increased in a time-dependent manner (Fig. 1B). To further investigate whether LPS-induced aldosterone secretion was through TLR2/TLR4, ZG cells were pretreated with TLR2/TLR4 blockers (0 and 10 μg/ml) for 1 h and then co-treated with LPS (0 and 10 μg/ml) for 12 h. Basal aldosterone release was unaffected by ZG cells treated with TLR2/TLR4 blocker alone, but LPS-enhanced aldosterone secretion was significantly suppressed ($P < 0.001$) by ZG cells treated with TLR2 or TLR4 blockers (Fig. 2A). Another TLR4 ligand, pure LPS, and specific TLR2 ligand, Pam3Cyc (Pam3) were used. ZG cells were treated with maximal dose of crude LPS (100 μg/ml), pure LPS (100 μg/ml), and Pam3 (10 μg/ml) for 12 h. Pure LPS and Pam3, similar to crude LPS, caused an approximately threefold increase in aldosterone secretion over the basal level (Fig. 2B; $P < 0.001$). These results indicated that LPS-induced aldosterone release was through both TLR2 and TLR4.

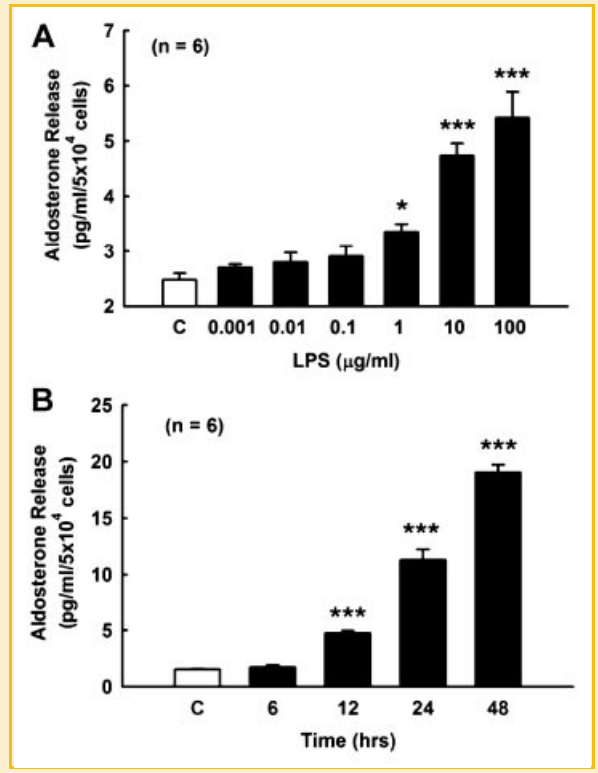

Fig. 1. LPS-induced aldosterone release by rat adrenal ZG cells. Dispersed ZG cells (5×10^4 cells/well/0.5 ml) were cultured in 24-well plates for 24 h then treated with LPS (0, 0.001, 0.01, 0.1, 1, 10, or 100 μg/ml) for 12 h (A). ZG cells were challenged with LPS (10 μg/ml) for 0, 6, 12, 24, and 48 h (B). Culture media was collected and aldosterone production was determined by RIA. Each bar represents the mean ± SEM. $^*P < 0.05$, $^{***}P < 0.001$ versus control group (n = 6).

LPS-INDUCED SR-B1, P450scc, AND STAR PROTEIN EXPRESSION PARTIALLY VIA TLR2 IN RAT ADRENAL ZG CELLS

To determine whether the stimulatory effects of LPS (10 μg/ml) on aldosterone release were caused by altered expressions of scavenger receptor-B1 (SR-B1; increases cholesterol content through HDL uptake), steroidogenic acute regulatory (StAR) protein (transfers cholesterol into mitochondria) and cytochrome P450 side chain cleavage enzyme (P450scc; transforms cholesterol into pregnenolone), the levels of those proteins in ZG cells were assessed by Western blotting. Based on the ratio of internal control, β-actin (45 kDa), the protein levels of SR-B1 (82 kDa; Fig. 3A), StAR (29 and 34 kDa; Fig. 3B) and P450scc (54 kDa; Fig. 3C) protein expression increased significantly by administration of LPS (10 μg/ml) for 12 h. To further study whether the stimulatory effects of LPS on aldosterone secretion were caused by altering the activity of aldosterone synthase, ZG cells were treated with deoxycorticosterone (DOC; 0 and 30 μM) and the aldosterone release significantly increased in comparison with control group, but this stimulatory effect was not changed by cells co-treated with LPS (Fig. 3D). This represented that the activity of aldosterone synthase was not affected by LPS.

ZG cells (1×10^6 cells) were pre-incubated with TLR2 or TLR4 blocker (anti-TLR4/MD-2 and anti-TLR2 antibody; 0 and 10 μg/ml)

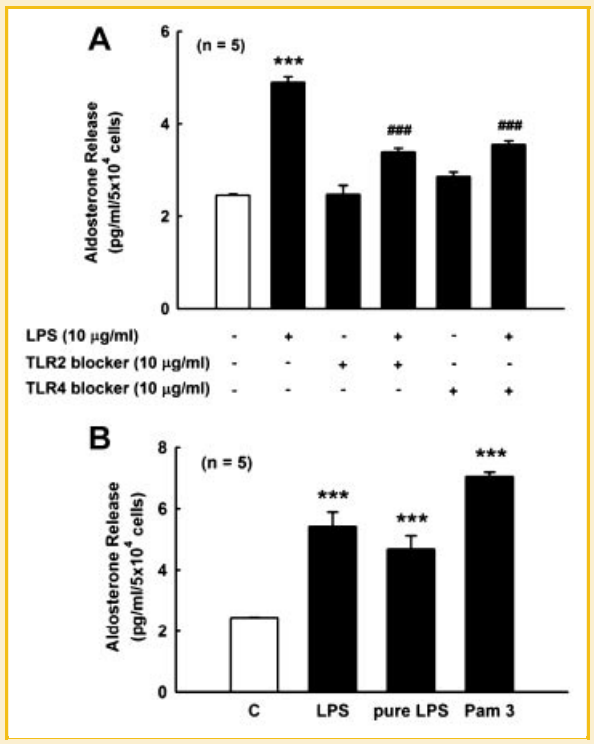

Fig. 2. LPS-induced aldosterone release via TLR2 and TLR4 in rat adrenal ZG cells. Dispersed ZG cells (5×10^4 cells/well/0.5 ml) were cultured in 24-well plates for 24 h. ZG cells were pretreated with TLR2/TLR4 blockers (0 and 10 μg/ml) for 1 h then co-treated with LPS (0 and 10 μg/ml) for 12 h (A). ZG cells were treated with LPS (100 μg/ml), pure LPS (100 μg/ml), and Pam3 (100 μg/ml) for 12 h (B). Culture media was collected and aldosterone production was determined by RIA. Each bar represents the mean ± SEM. $^{***}P < 0.001$ versus control group; $^{###}P < 0.001$ versus LPS alone (n = 5).

for 1 h and then challenged with LPS (0 and 10 μg/ml) for 12 h, the SR-B1, P450scc, and StAR protein expression was assessed by Western blotting (Fig. 4A). The results showed that LPS-induced SR-B1 (Fig. 4B) and StAR (Fig. 4C) protein expressions were abolished by cells treated with TLR2 blocker. LPS-enhanced SR-B1 protein expression was partially abolished by TLR4 blocker treatment (Fig. 4B, $P > 0.05$), and LPS-increased StAR protein expression was not affected by TLR4 (Fig. 4C). Therefore, LPS-stimulated SR-B1 and StAR protein expressions were through TLR2.

EFFECTS OF LPS ON AKT PHOSPHORYLATION

ZG cells were treated with LPS (0 and 10 μg/ml) for 0, 5, 15, 30, or 60 min. Images from a confocal microscope showed that LPS-induced Akt phosphorylation was mainly located in the cytoplasm (Fig. 5A, medium panel, red fluorescence). DAPI was used to label nuclei (Fig. 5A, lower panel, blue fluorescence). ZG cells were challenged with LPS (0 and 10 μg/ml) for 0, 1, 3, 5, 10, 15, or 30 min. Therefore, LPS (10 μg/ml) could significantly increase Akt phosphorylation. Maximal response reached after LPS treatment for 15 min and declined to basal levels after 30 min (Fig. 5B). Basal Akt phosphorylation in ZG cells was unaffected by a 1-h pretreatment with TLR2, TLR4 blockers (0 and 10 μg/ml) or 5 μM LY294002 (an inhibitor of PI_3K) (Fig. 5C). LPS-induced Akt phosphorylation was suppressed by ZG cells treated with blockers of TLR2, TLR4 (10 μg/ml) or LY294002 (5 μM) for 15 min (Fig. 5C). Our results suggested that Akt phosphorylation is enhanced by LPS treatment via TLR2, TLR4, and PI_3K pathway.

LPS-INDUCED ALDOSTERONE PRODUCTION AND SR-B1 PROTEIN EXPRESSION VIA PI_3K/Akt SIGNALING PATHWAYS

ZG cells (5×10^4 cells/well) were pre-incubated with LY294002 (0, 5, and 25 μM) for 1 h. Then, they were co-treated with LPS (0 and 10 μg/ml) for 12 h. LPS-stimulated aldosterone secretion from ZG cells was markedly abrogated by being pre-incubated with LY294002 (5, 25 μM; Fig. 6A). These results suggest that LPS-induced aldosterone secretion is regulated by the PI_3K/Akt signaling pathways.

ZG cells were pre-incubated with LY294002 (5 μM) for 1 h and then co-treated with LPS (0 and 10 μg/ml) for 12 h. Pre-incubation with LY294002 significantly abrogated LPS-induced SR-B1 protein expression, but not StAR and P450scc proteins expression (Fig. 6B,C).

DISCUSSION

High level of plasma aldosterone is an indicator of cardiovascular disease [Rossi et al., 2005]. Our studies have demonstrated that LPS could directly increase aldosterone production for 48 h in rat adrenal ZG cells. Our results provided the first evidence that LPS-induced aldosterone production is through TLR2 and TLR4 related PI_3K/Akt signal transduction pathways in adrenal ZG cells.

Previous studies have established that LPS, a bacterial endotoxin, activates inflammatory response or sepsis seriously. LPS enhances the expression of inflammatory mediators such as TNF-α and IL-6, and it also elevates aldosterone levels in both the serum and the adrenal glands [Path et al., 1997; Sanchez-Lemus et al., 2008]. These in vivo studies cannot distinguish the signal transduction pathways of aldosterone or corticosterone secretion in adrenal cortex. Aldosterone is secreted from adrenal ZG cells. Therefore, we identified pure ZG cells from rat adrenal gland by primary culture to investigate the mechanisms of aldosterone production. Our studies showed that aldosterone release keep increasing even after 48 h of LPS treatment (1–100 μg/ml) (Fig. 1A). But this long-term effect does not exist in adrenal zona fasciculate-reticularis cells secreting corticosterone (data not shown). Therefore, the mechanisms of LPS-stimulated aldosterone production are more important in cardiovascular disease.

During inflammation or endotoxemia, the hypothalamic-pituitary-adrenal axis and renin-angiotensin-aldosterone system are markedly activated. In this response, plasma ACTH and Ang II play an important role in enhancing aldosterone production [Garcia et al., 1990; Givalois et al., 1994]. Sanchez-Lemus et al. [2008] demonstrated that the AT_1R blocker candesartan decreased LPS-induced plasma aldosterone concentration in the first hour, but it cannot completely block the rise of aldosterone levels in plasma during LPS administration. Therefore, LPS-induced aldosterone secretion may not be totally mediated via systemic effects. Our study

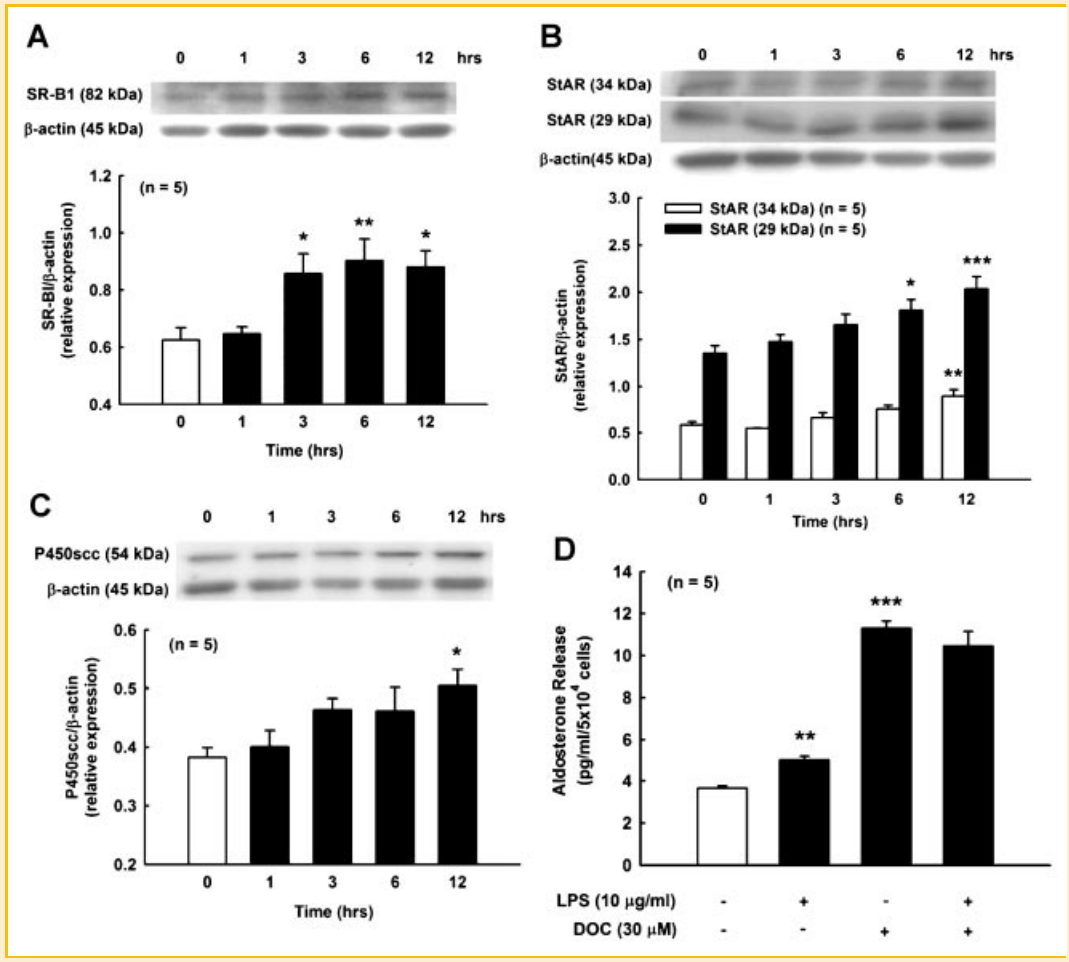

Fig. 3. LPS induces the proteins expression for steroidogenesis in rat adrenal ZG cells. After 3 days of culture, ZG cells (1×10^6 cells) in 6 cm petri dishes were treated with LPS (0 and 10 μg/ml) for 1 to 12 h to determine SR-B1 (A), StAR (B), and P450scc (C) protein levels. Cells were collected and their proteins were extracted. SR-B1, StAR, and P450scc protein levels in the cell lysate were analyzed by Western blotting analysis, and β-actin used as an internal control. Bands were quantified by Image Quant software and SR-B1, StAR, and P450scc protein levels were normalized to β-actin. ZG cells were treated with LPS (0 and 10 μg/ml) or combined with deoxycorticosterone (DOC; 0 and 30 μM) for 12 h, the aldosterone release was detected by RIA (D). Each bar represents mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group (n = 5).

is the first to distinguish the direct effect of LPS and its important role in aldosterone production.

Several studies have shown that the relationship between the immune and endocrine systems is vital to maintain homeostasis in several illnesses [Beishuizen and Thijs, 2003]. TLRs are responsible for promoting the immune response, and it has been shown that TLR2 widely recognizes bacteria and other components of different pathogens, and TLR4 has been implicated in LPS signaling, innate immunity, and inflammation [Hajjar et al., 2001]. They were recently identified in human and mouse adrenal glands and played a role in enhancing NF-κB-mediated inflammatory mediators in the adrenal gland such as TNF-α, IL-6, and IL-8 [Bornstein et al., 2004a,b; Zacharowski et al., 2006; Kanczkowski et al., 2009]. The specific ligands for TLR2 (Pam3) and TLR4 (pure LPS and lipid A) were found to stimulate cortisol release, suggesting that these receptors may mediate the effects of LPS in adrenal cells, as had been showed in other cell types [Vakharia et al., 2002; Vakharia and Hinson, 2005]. In adrenal ZG cells, our study demonstrated that anti-TLR2 and anti-TLR4 can reduce crude LPS-induced aldosterone production (Fig. 2A). However, previous study reported that TLR2 activation by crude LPS is mediated by contaminants, such as lipoprotein [Hirschfeld et al., 2000]. We used another TLR4 ligand (pure LPS) and specific TLR2 ligand (Pam3CSK4) to demonstrate that aldosterone still increases (Fig. 2B). In addition, TLR2 or TLR4 siRNA were transfected into adrenal ZG cells to examine whether the expression of TLR2 or TLR4 were the essential factor in aldosteron production. The result indicated that LPS-induced aldosterone production was significantly reduced by TLR2 or TLR4 siRNA treated (data not shown). Therefore, these two receptors play an important role on bacterial-induced aldosterone production. In vivo studies have showed that TLRs in the adrenal gland modulate glucocorticoid secretion in response to stress through NF-κB [Bornstein et al., 2006; Kanczkowski et al., 2008], and TLR2-deficient mice failed to respond properly when they were treated with LPS and lipoteichoic acid (Gram-positive bacterial cell wall components) [Bornstein et al., 2004b; Zacharowski et al., 2006]. However, the mechanisms of LPS-stimulated aldosterone production in normal adrenal gland cells remain unclear.

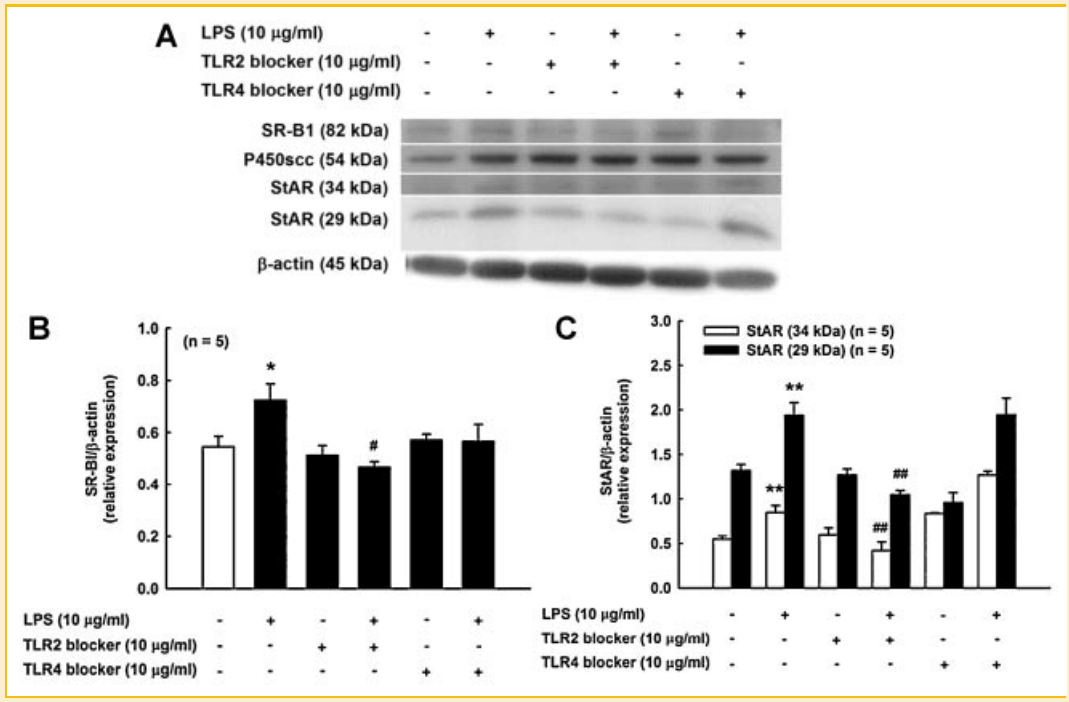

Fig. 4. LPS induces the SR-B1 and StAR protein expression via TLR 2 in rat adrenal ZG cells. After 3 days of culture, ZG cells (1×10^6 cells) in 6 cm petri dishes were pre-incubated with TLR2 or TLR4 blockers (0 or 10 μg/ml) for 1 h then treated with LPS (0 and 10 μg/ml) for 12 h to determine SR-B1, P450scc, and StAR protein expression by Western blotting analysis (A). The statistic data of SR-B1 (B) and StAR protein (C) were showed below. Cells were collected and their protein was extracted. SR-B1, StAR, and P450scc protein levels in the cell lysate were analyzed via Western blotting analyses, and β-actin was used as an internal control. Bands were quantified by Image Quant software and SR-B1, StAR and P450scc protein levels were normalized to β-actin. Each bar represents mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the basal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LPS alone (n = 5).

Cholesterol is a precursor of adrenal steroid hormones, and more than 80% of adrenal cholesterol is derived from lipoprotein uptake [Andersen and Dietschy, 1978; Gwynne and Strauss, 1982; Kraemer, 2007]. The major source of cholesterol is from the uptake of circulating HDL by SR-B1 [Rigotti et al., 1997; Temel et al., 1997]. In steroidogenesis, the initial and rate limiting step is StAR protein, which transfers free cholesterol to the inner mitochondrial membrane. This membrane, P450scc remodels the cholesterol molecules to convert them to pregnenolone [Burstein and Gut, 1976; Christenson and Strauss, 2000]. The production of aldosterone, which includes the LDL receptor, SR-B1, the HMG-CoA receptor, SCAP, P450, and StAR proteins, has been reported to be enhanced by LPS administrated in the adrenal gland [Sanchez-Lemus et al., 2008]. The LPS-promoting effect on aldosterone production occurs with a concomitant arrest in steroidogenesis. Our studies indicated that LPS increased in SR-B1 and StAR protein levels and a transient increase in P450scc protein expression (Fig. 3A–C). And this increase of SR-B1 and StAR protein level was also reduced by TLR2 blocker. It clarified that TLR2 played an important role in aldosterone stress response and its steroidogenesis processes, such as SR-B1 and StAR protein expression (Fig. 4A-C). The final step in aldosterone biosyntheses is the conversion of corticosterone to aldosterone under the catalyzation of the enzyme aldosterone synthase. We found that DOC (30 μM) induced aldosterone secretion is not affected by LPS (10 μg/ml). These data suggest that the stimulatory effect of LPS on aldosterone secretion was not related to the activity of aldosterone synthase.

The functional role of PI_3K/Akt in the inflammatory response in immune cells was worth discussion. The PI_3K/Akt signaling pathway has been shown to play an important role in negatively regulating LPS-induced acute inflammatory responses in vitro and in vivo [Kim et al., 2002; Bommhardt et al., 2004; Zhang et al., 2007]. Therefore, our data are consistent with previous findings that LPS induces Akt phosphorylation through the activated PI_3K and TLR2 in adrenal ZG cells (Fig. 5). Thus, it appears that the delay in the activation of PI_3K/Akt initially allows LPS to activate an acute inflammatory response, which is subsequently blunted and shut down by LPS-induced Akt phosphorylation [Fukao and Koyasu, 2003; Zhang et al., 2007]. These processes constitute an important mechanism in the adrenal response to LPS.

PI_3K/Akt pathway, which is act upstream of NF-κB, is also activated by ROS in human monocytic cells [Ozes et al., 1999; Han et al., 2001; Kim et al., 2008b]. Furthermore, it is well documented that NF-κB plays a prominent role in LPS-induced activation and transcriptional regulation of most inflammatory genes [Griendling et al., 2000; Asehnoune et al., 2004; Jang et al., 2006; Liu and Malik, 2006]. Indeed, the adrenal gland is an important organ in the production of steroid hormones, and the PI_3K/Akt pathways also play a decisive role in steroidogenesis. So far, there has been no evidence concerning the TLR pathways to aldosterone production. Of note, however, PI_3K has been implicated in the stimulation of

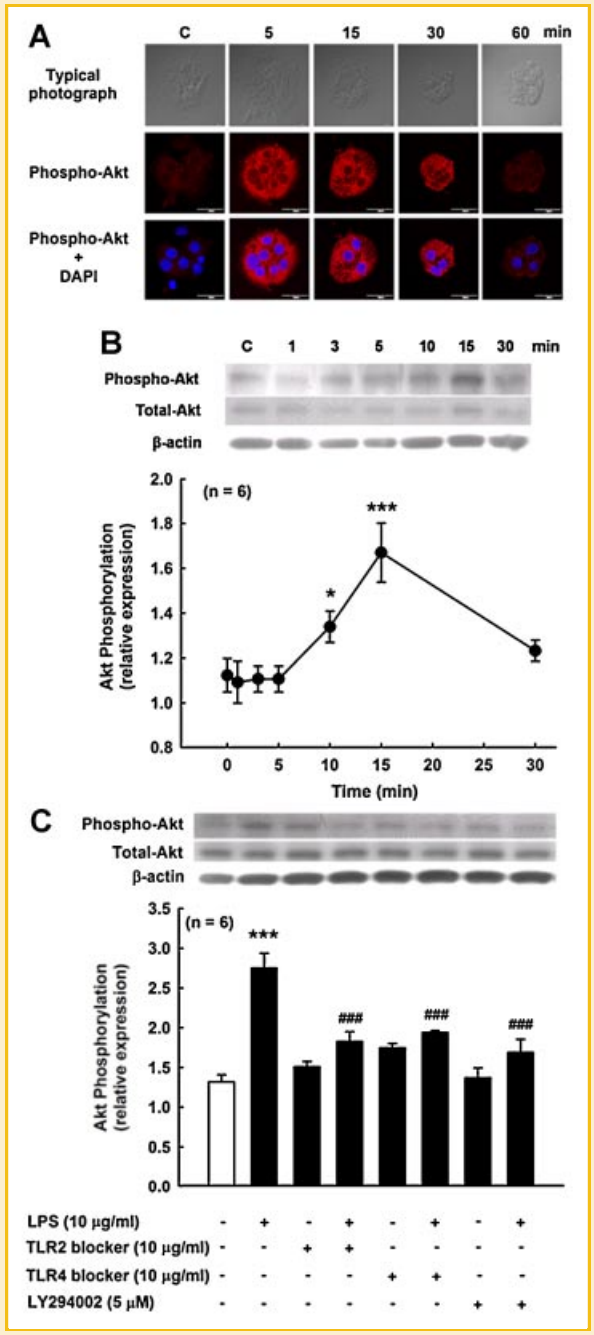

Fig. 5. Effects of LPS on Akt phosphorylation in rat adrenal ZG cells. ZG cells (5×10^4 cells/0.5 ml) were stimulated with LPS (0 and 10 μg/ml) for 0–60 min. Phosphorylated Akt (Phospho-Akt) expression (medium panel, red fluorescence) and DAPI (lower panel, blue fluorescence, DNA dye) are shown in confocal immunofluorescent images (A). ZG cells (1×10^6 cells) in 6 cm petri dishes were treated with LPS (0 and 10 μg/ml) for 0–30 min (B). ZG cells were pre-incubated with or without 10 μg/ml TLR2, TLR4 blocker or 5 μM LY294002 (an inhibitor of PI_3K) for 1 h before being treated with LPS (0 and 10 μg/ml) for 15 min (C). Then, quantities of phospho-Akt, total-Akt and β-actin in cell lysates were determined by Western blotting analyses with β-actin as an internal control. Bands were quantified by Image Quant software and phosphorylated Akt was normalized to total Akt expression. Each value represents the mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ versus basal group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus LPS alone (n = 6).

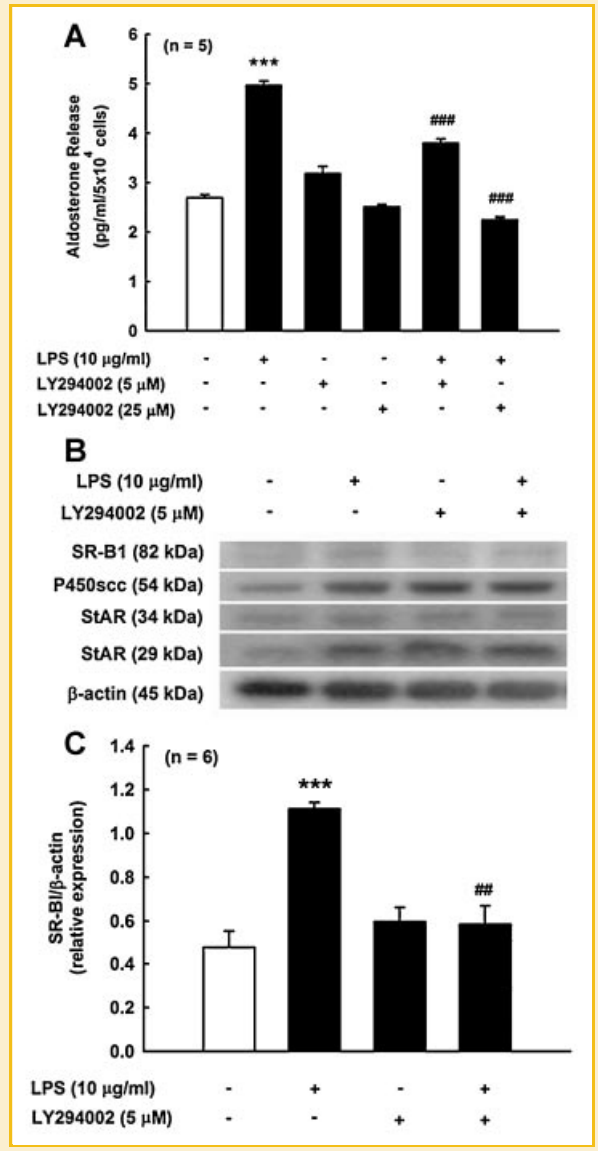

Fig. 6. LPS-induced aldosterone production and SR-B1 protein expression via the PI_3K/Akt pathways. ZG cells (5×10^4 cells/0.5 ml) were cultured in 24-well plates for 24 h. ZG cells were pre-incubated with LY294002 (0, 5, and 25 μM) for 1 h then treated with LPS (0 and 10 μg/ml) for 12 h. Culture media were collected and aldosterone production determined by RIA (A). Moreover, ZG cells (1×10^6 cells) were pre-incubated with or without 5 μM LY294002 (a potent inhibitor of PI_3K) for 1 h then treated with LPS (0 and 10 μg/ml) for 12 h to determine SR-B1, P450scc and StAR protein expression (B). Bands were quantified by Image Quant software and SR-B1 protein levels were normalized to β-actin (C). Each bar represents mean ± SEM. $^{***}P < 0.001$, compared with the basal group; $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with LPS alone (n = 6).

aldosterone production and steroidogenesis by different agonists, such as Ang II and ACTH [Campbell et al., 2003], in adrenal glomerulosa cells [Meroni et al., 2002; Banno et al., 2003; Spat, 2004; Shah et al., 2005]. Therefore, our data demonstrated that the PI_3K/Akt pathways may regulate not only inflammatory processes in the adrenal gland, but also the related aldosterone production (Fig. 6A). It also plays a critical role on SR-B1 proteins,

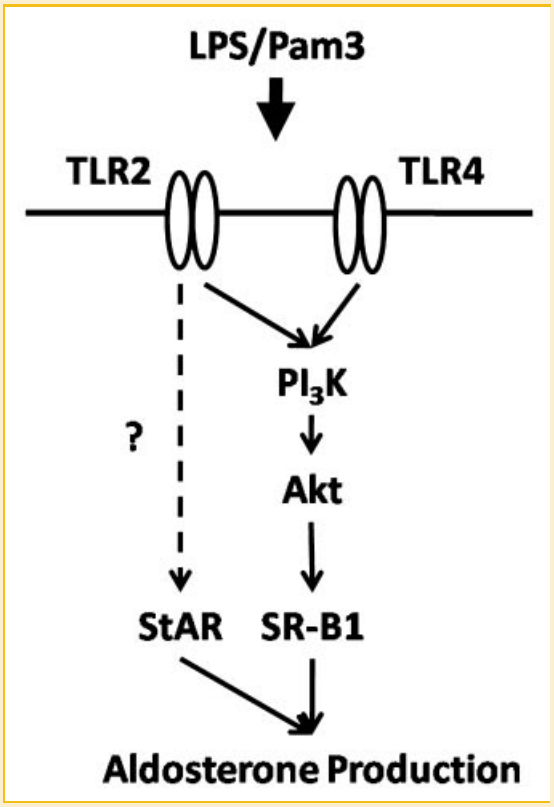

Fig. 7. Proposed model for the mechanism of LPS-induced aldosterone production mediated by the TLR2 and TLR4 related PI_3K/Akt signal transduction pathways.

which is one of important steroidogenesis process in adrenal gland (Fig. 6C). However, another steroidogenesis protein, StAR protein, also increases via TLR2 during LPS treatment, but PI_3K/Akt pathway is not involved in this process. It is worth investigating the mechanism of TLR2-regulated StAR protein expression.

In conclusion, our study provided the information about aldosterone synthesis during LPS treatment in adrenal ZG cells. Therefore, SR-B1 and StAR protein could be the one of the important downstream proteins in the TLR2 signal transduction pathway. Besides, LPS can act TLR2 and TLR4 to activate PI_3K/Akt pathways and then increases aldosterone production and SR-B1 protein levels (Fig. 7). It is possible that overproduction of aldosterone may recover during down regulation of the TLR2, TLR4, PI3K/Akt pathway, SR-B1, and/or StAR function. Besides, LPS-induced aldosterone production significantly increased until 24 and 48 h, and the long-term regulation of LPS in adrenal gland could be discussed further.

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