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Reduction of TLR4 mRNA Stability and Protein Expressions through Inhibiting Cytoplasmic Translocation of HuR Transcription Factor by E_2 and/or ERα in LPS-Treated H9c2 Cardiomyoblast Cells

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Abstract

Our previous results have indicated that Akt mediates 17β-estradiol (E_2) and/or estrogen receptor α (ERα) to inhibit lipopolysaccharide (LPS)-induced JNK activity, tumor necrosis factor α (TNFα) protein expression, and exhibits cardioprotective effects. Toll-like receptor 4 (TLR4) mRNAs often contain AU-rich elements (AREs) in their 3'-untranslated regions (3'UTR) which have a high affinity for RNA-binding proteins. It is not known whether E_2 and ERα affect TLR4 mRNA stability and TLR4 protein expression through regulating the RNA-binding proteins, human antigen R (HuR), tristetraprolin (TTP) and AU-binding factor 1 (AUF-1) in myocardial cells. Therefore, we investigated if the LPS induces these RNA-binding proteins to regulate TLR4 mRNAs of cardiomyocytes, and whether the E_2/ERα reduces the TLR4 mRNA stability induced by LPS through the inhibition of RNA-binding protein expression. Using a doxycycline (Dox)-induced Tet-On ERα H9c2 myocardic cell model, we also aimed to identify whether E_2 and/or ERα regulate LPS-induced TLR4 mRNA stability. The results of Western blotting and reverse transcription-PCR assays demonstrated that LPS significantly increased the level of cytoplasmic HuR protein and the stability of TLR4 mRNA, and farther induced TLR4 protein expression in H9c2 cells, an effect mediated through the JNK pathway. Interestingly,

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E_2 and/or ERα decreased the cytoplasmic HuR protein level and TLR4 mRNA stability, and farther decreased the level of TLR4 protein induced by LPS in H9c2 cardiomyoblast cells. Therefore, LPS triggered HuR expression which led to enhanced TLR4 mRNA and upregulated TLR4 expression through JNK1/2 in myocardial cells.

Key Words: E_2, ERα, HuR, H9c2 cardiomyoblast cells, LPS

Sepsis is a systemic physiological and pathological response to severe inflammation. The inflammatory response to infection or injury is a highly conserved and regulated reaction of the organism (10). Previous research has indicated that endotoxin, such as lipopolysaccharide (LPS), is an important pathogen responsible for cardiovascular disorders (18). LPSs are high-molecular-weight complexes that are major components of the outer membranes of the cell wall of gram-negative bacteria. LPS is composed of oligosaccharide domains and a lipid A domain. The oligosaccharide domains contain two subunits: O-specific side chain and core oligosaccharide. Lipid A, the anchor moiety of LPS, is the active component for its toxic activity to bind to Toll-like receptor 4 (TLR4) (33). TLRs are type I transmembrane receptors that are expressed on the cell membrane after stimulation. LPS-induced TLR4 expression on the surface of cardiacmyocytes plays a role in cardiac dysfunction. TLR4 mediates myocardial dysfunction *via* NFκB-dependent mechanisms through an increase in tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) production by themselves. However, TLR4 inhibition reduces IL-1β, NFκB, and TNFα pathways to improve cardiomyocyte contractility after LPS application (8).

Epidemiological researches show that pre-menopausal women have lower rates of cardiovascular diseases than age-matched men. However, women after menopause have higher morbidity rates but estrogen-replacement therapy contributes to low incidences of heart disease (5). Steroid estrogen containing estrone (E_1), 17β-estradiol (E_2), and estriol (E_3). E_2 is the principle intracellular human estrogen and is more potent than estrone and estriol and is the primary estrogen secreted prior to menopause. E_2 modulates cell proliferation and differentiation, development of the reproductive system such as uterus, vagina, breast development, male testis, epididymis, the prostate, maintain bone density of skeletal system, and protect the cardiac system (28). E_2 signaling is transduced though estrogen receptors (ERs) which are members of the superfamily of steroid/thyroid hormone nuclear receptors (30). ERs are composed of six functional domains, termed A to F. The N-terminal, A/B domain is an agonist-independent transcriptional activation function domain-1 (AF-1) which can turn on transcription without estrogen binding. The C domain is a DNA-binding domain (DBD). The D domain is the hinge domain, which links the C and E domains. The E domain is the ligand-binding domain (LBD) which can encompass both an agonist-dependent transcriptional activation function domain-2 (AF-2) and a dimerization region. The F domain is important for modulating transactivation and protein-protein interactions (19). The molecular mechanisms of estrogen signaling can be ligand-dependent, ligand-independent, DNA binding-independent or nongenomic signalling (16).

Mitogen activated protein kinases (MAPKs) including extracellular signal regulated kinase (ERK), p38 MAPK and c-jun amino-terminal kinase (JNK) play important roles in cellular functions. In myocardial cells, ERK1/2 protects the cells from apoptosis in ischemia and redox stress (42). ERKs modulate transcription of inflammatory genes by up-regulation of AP-1 components (36). The p38 MAPK is referred to pro-apoptotic and anti-apoptotic actions. Chronic treatment with a p38 MAPK inhibitor, SB239063, prevents left ventricular (LV) hypertrophy and dysfunction in hypertensive rats (9). Studies show that the JNK signaling pathway contributes to the regulation of cell proliferation and apoptosis (15). JNK reverse cell apoptosis and retards cell death (1).

Protein expression may operate with multiple mechanisms, such as transcription, translation, and mRNA degradation. Cytokines and chemokines usually have low levels of expression in non-stimulated cells (6), and increase mRNA levels and translation of mRNA during inflammatory responses. Unstable mRNAs often contain AU-rich elements (AREs) in their 3'-untranslated region (UTR) (11). The characteristic motif of AREs is AUUUA, but the number of copies of ARE and AU contents is highly variable (20). There are three classes of AREs: Class I contains one to three scattered AUUUA motifs located near U-rich sequences; Class II contains multiple clusters of AUUUA copies. Class III ARE does not contain the AUUUA motif but mediates mRNA degradation (35, 39). AREs are functionally separated by their ability to confer instability to otherwise stable mRNAs. The regulatory function of AREs is mediated through the RNA-binding proteins that recognize the ARE motifs (11, 12, 22), including human antigen R (HuR), AU-binding factor 1 (AUF1), and the zinc-finger protein tristetraprolin (TTP). HuR is a member of the embryonic lethal abnormal vision (ELAV) family of RNA-

binding proteins which shuttle between the nucleus and cytoplasm and predominantly exist in nuclear proteins.

HuR binds strongly to AREs and stabilizes the bound mRNAs (22). HuR does not affect deadenylation but delays the commencement of decay of the RNA body and slows down its subsequent decay (32). AUF1 is a member of the heteronuclear ribonucleoprotein (hnRNP) family which exists in four isoforms with 37, 40, 42, and 45 kDa molecular weights. AUF1 isoforms have different roles of mRNA turnover (32). Overexpression of AUF1 may destabilize (25) or stabilize AREs (40). TTP, a zinc-finger proteins, is critically implicated in inflammation that binds to AREs and destabilizes TNFα mRNAs (21). It hinders both the deadenylation and decays of the mRNA body (26). Our previous results have indicated that LPS induces myocardiac cell hypertrophy, apoptosis and fibrosis. We also found that E_2 and estrogen ERα exhibit cardioprotective effects by inhibiting JNK1/2-mediated LPS-induced TNF-α expression and cardiomyocyte apoptosis through activation of Akt (23). Here, we aimed to further investigate if LPS induces the RNA binding protein, human antigen R (HuR), to regulate TLR4 mRNAs, which contain AU-rich element (ARE) in 3'UTR in cardiomyocytes. Additionally, using a doxycycline (Dox)-induced Tet-On ERα H9c2 myocardiac cell model, we also aimed to investigate whether E_2 and ERα manipulate the LPS-induced TLR4 mRNA stability.

Materials and Methods

Cell Culture

Heart-derived H9c2 myocardial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). H9c2 cells and Tet-On/ERα H9c2 cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin in 5% CO_2 humidified air at 37°C. Media were replaced 2~3 times a week. H9c2 cells were cultured in serum-free medium with minimal essential medium for 12 h for drug treatment. The incubation was continued for 24 h, and then the cells were harvested and extracted for analysis.

Total RNA Extraction

Cells were lysed directly in a culture dish by adding the Ultraspec RNA and passing the cell lysate several times through a pipette. The cell lysate were transferred immediately into centrifuge tubes. The cell were stored for 5 min at 4°C to permit complete dissociation of nucleoprotein complexes. Next, 0.2 ml of chloroform was added per 1 ml of Ultraspec RNA, the samples were covered tightly and shaken vigorously for 15 sec and placed on ice for 5 min. The homogenate was centrifuged at 12,000 × *g* at 4°C for 15 min. The aqueous phase (40~50%) was carefully transferred to a fresh tube while taking care not to disturb the interphase. Equal volume of isopropanol and samples were stored for 10 min at 4°C. Samples were centrifuged at 12,000 × *g* (4°C) for 10 min. The supernatant was removed and the RNA pellet was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 × *g* at 4°C. The RNA pellet was dissolved in 50-100 μl DEPC-treated water or in an appropriate buffer by vortexing for 1 min. The RNA was quantified and checked for purity and condition by spectrophotometry at 260 nm.

Reverse Transcription (RT) and Polymerase Chain Reaction Amplification

To 59.5 μl DEPC-H_2O containing 8 g RNA, 0.5 μl RNase inhibitor (N251B; Promega, WI, USA) (40 U/μl, 20 μl 5X RT buffer (N251B; Promega) (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 8 μl dNTP (4030; Takara Bio Inc., Shiga, Japan) (2.5 mM), and 10 μl oligo-dT (10 mM) were added. Reaction was initiated at 70°C for 5 min, and 2 μl RTase (M368B; Promega) was added after 5 min. The samples were then incubated at 42°C for 1 h, 95°C for 5 min, and stored at 4°C. MMLV Reverse transcriptase (M170B; Promega) MMLV Reverse 5×buffer (M531A; Promega), recombinant RNasin Ribonuclease inhibitor (N251A; Promega), dATP, dCTP, dTTP and dGCP (Promega), oligo-dT (Mission Biotech, Taipei, Taiwan, ROC), Taq DNA polymerase (611069; MD Bio, Taipei, Taiwan, ROC) and 10×PCR buffer (708059; MD Bio) were added.

For PCR, five microliter RT product was diluted with the PCR buffer (50 mM KCl, 10 mM Tris-HCl and 2 mM $MgCl_2$), adding 0.5 M dNTPs (final concentration, 0.8 mM) and 0.5 U of Taq DNA polymerase to a final volume of 50 μl. PCR was initiated with a hot start (5 min at 95°C); the samples were then subjected to 32 cycles at 95°C for 1 min, annealing temperature for 1 min, and 72°C for 2 min. The annealing temperature for the TLR4, and TNFα primers was 58°C; the GAPDH primers was 55°C. This was followed by a final extension step at 72°C for 20 min, and store at 4°C. Primers were as follows: rat GAPDH forward primer: GGGTGTGAACCACGAGAAAT, reverse primer: CACAGTC TTCTGAGTGGCA; rat TNF-α forward primer: CCTCTTCTCATTCCTGCTCG, reverse primer: GGTATGAA ATGGCAAATCGG; TLR4 forward primer: CATGGCATTGTTCCTTT- CCT, reverse primer: CATGGAGCCTAATTCCCTGA. Ten microliter product mixed with 2 μl 6X loading dye was as-

sessed by 1.5% agarose gel electrophoresis and DNA was visualized by ethidium bromide staining.

Nuclear Extraction

Cells were re-suspended with 200 μl ice-cold buffer-I (10 mM Hepes, pH 8.0; 1.5 mM $MgCl_2$; 10 mM KCl; 1 mM dithiothreitol and proteinase inhibitor cocktail) (04-693-H9-001; Roche, Sandhoferstrasse Mannheim, Germany) and incubated for 15 min on ice to allow cells to swell, followed by addition of 20 μl IGEPAL-CA630. The homogenate was centrifuged at 14,000 × *g* at 4°C for 15 min. The cytoplasmic fraction was carefully aspirated. The pellet was re-suspended with ice-cold Buffer-II (20 mM Hepes, pH 8.0; 1.5 mM $MgCl_2$; 25% glycerol; 420 mM NaCl; 0.2 mM EDTA; 1 mM dithiothreitol and proteinase inhibitor cocktail) (Roche) and vigorously vortexed. The homogenate was centrifuged at 14,000 × *g* at 4°C for 15 min. The supernatants (nuclear extracts) were collected. Cytoplasmic fraction and nuclear extracts were stored at -80°C. The protein concentration was determined by colorimetric assay (Bio-Rad, Hercules, CA, USA).

Western Blotting

Cells were re-suspended in lysis buffer (50 mM Tris, pH 7.5; 0.5 M NaCl; 1.0 mM EDTA, pH 7.5; 10% glycerol; 1 mM BME; 1% IGEPAL-630) and a proteinase inhibitor cocktail (04-693-H9-001; Roche). Samples containing equal amounts of protein (40 μg) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (IPVH000 10, Millipore, Billerica, MA, USA). The membranes were incubated with the blocking buffer in a shaker for 1 h and then incubated overnight with primary antibodies (dilution, 1:1,000) at 4°C overnight. The primary antibodies used in this study included: anti-AUF-1 (ARP40230; Aviva Systems Biology, San Diego, CA, USA), HDAC5 (sc-11419; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-HuR (sc-20694; Santa Cruz Biotechnology, Inc.), anti-IL-6 (sc-1266; Santa Cruz Biotechnology, Inc.), anti-TNF-α (sc-1350; Santa Cruz Biotechnology, Inc.), anti-TLR4 (sc-16240; Santa Cruz Biotechnology, Inc.), anti-TTP (sc-14030; Santa Cruz Biotechnology, Inc.), and anti-α-Tubulin (sc-5286; Santa Cruz Biotechnology, Inc.). Membranes were washed three times with TBS and then incubated with horseradish peroxidase-conjugated secondary antibodies. The following secondary antibodies were used in this study: anti-goat-HRP (sc-2354; Santa Cruz Biotechnology, Inc.), anti-mouse-HRP (sc-2005; Santa Cruz Biotechnology, Inc.), and anti-rabbit-HRP (sc-2004; Santa Cruz Biotechnology, Inc.). The membranes were washed three times with TBS. The proteins of interest were visualized by using the substrate buffer and were detected by exposure to X-ray films.

Immunofluorescence

H9c2 cells subjected to various treatments were subsequently fixed with 4% paraformaldehyde at room temperature for 30 min. Cells were permeabilized with 0.5% Triton X-100 for 10 min at 4°C. The fixed cells was blocked with PBS containing 2% bovine serum albumin at 37°C for 30 min, followed by incubation with DAPI and the primary HuR antibody overnight at 4°C. After washing, cells were incubated with an anti-rabbit FITC-conjugated antibody at 37°C for 1 h. Fluorescence was visualized using a fluorescence microscope coupled with an image analysis system.

Statistical Analysis

All experiments were repeated at least three times using independent culture preparations. Values are shown as mean ± SEM. Student's *t*-test was used to calculate the statistical significance of the experimental results for two groups; a P value of < 0.05 was considered significant.

Results

LPS Dose-Dependent Up-Regulation of TLR4 Transcription and Protein Expression

To investigate whether LPS can regulate TLR4 expression in myocardial cells, Tet-On/ERα H9c2 myocardial cells were pretreated for 24 h with 0-1.0 μg/ml of LPS, and TLR4 mRNA and protein were analyzed by semi-quantitative PCR and Western blot analysis, respectively. Total RNA and protein were then extracted from the H9c2 cells. Results showed that inhibiting HuR in LPS-treated H9c2 cells, LPS dose-dependently up-regulated TLR4 mRNA expression and maximally elevated TLR4 mRNA at 1.0 μg/ml dosage. Similarly, LPS pretreatment significantly induced TLR4 protein expression in a dose-dependent manner (Fig. 1B).

LPS-Mediated TLR4 mRNA and Protein Expression in H9c2 Cardiomyoblast Cells Was Time-Dependent

H9c2 cadiomyoblast cells were treated with vehicle or LPS (1 μg/ml) for 0, 4, 12, 24 or 28 h. Total RNA was extracted and analyzed by RT-PCR. LPS significantly induced TLR4 and TNFα mRNA expressions. The expression of TLR4 and TNFα mRNA reached a maximum at 24 h after stimulation with LPS (Fig.

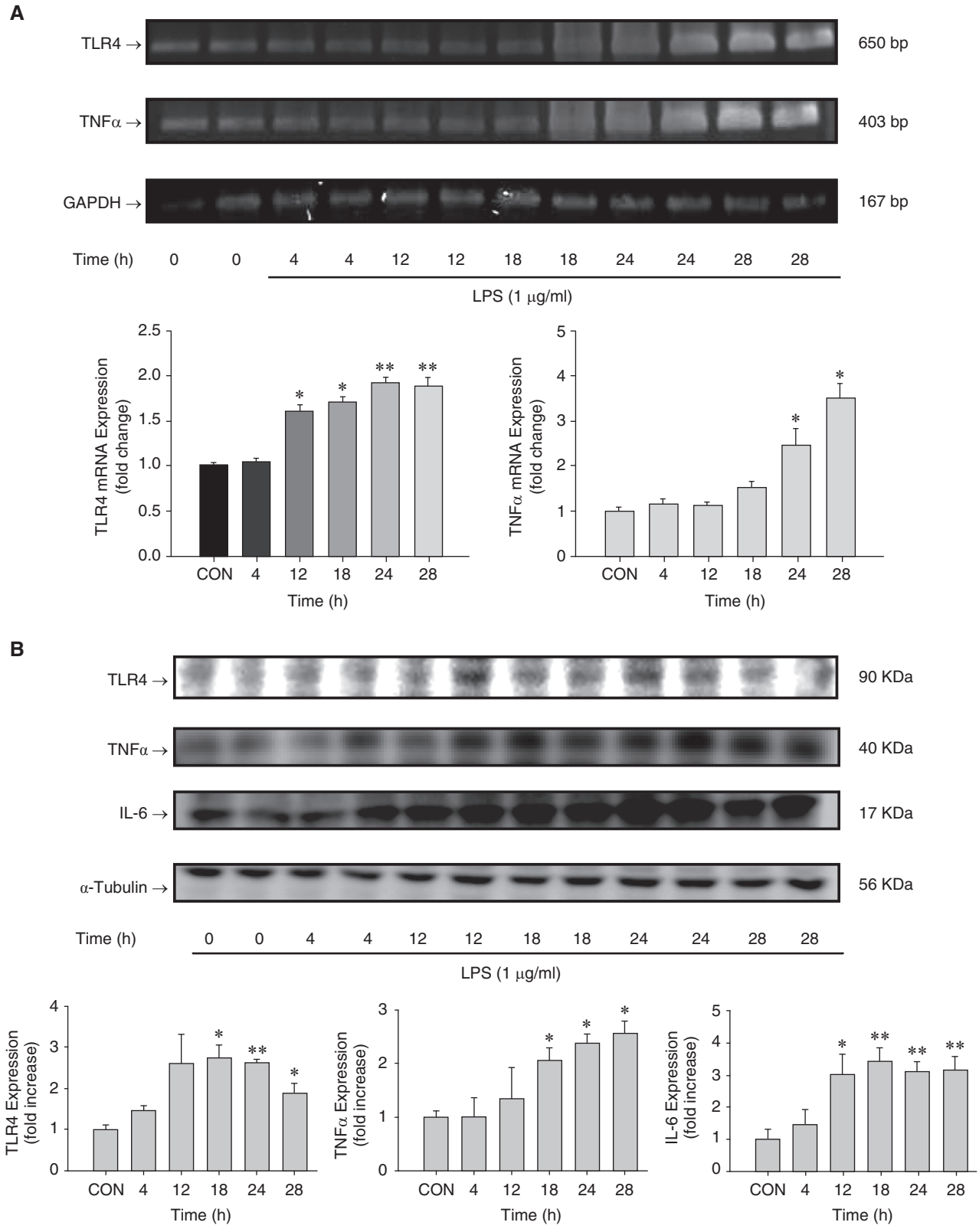

Fig. 1. LPS up-regulates TLR4 and TNFα mRNA transcription and protein expression in a dose-dependent manner. (A) Tet-On/ERα H9c2 cells were incubated for 24 h with the indicated concentrations of LPS (0-1.0 μg/ml). TLR4 and TNFα transcription was analyzed by RT-PCR after normalization to GAPDH mRNA. (means ± SEM; *$P < 0.05$, **$P < 0.01$, compared with unstimulated group) (B) Cell extracts were analyzed by Western blotting with antibodies against proteins as indicated (relative to α-tubulin). Data represent the results of three independent experiments (means ± SEM; *$P < 0.05$, **$P < 0.01$ compared to unstimulated group).

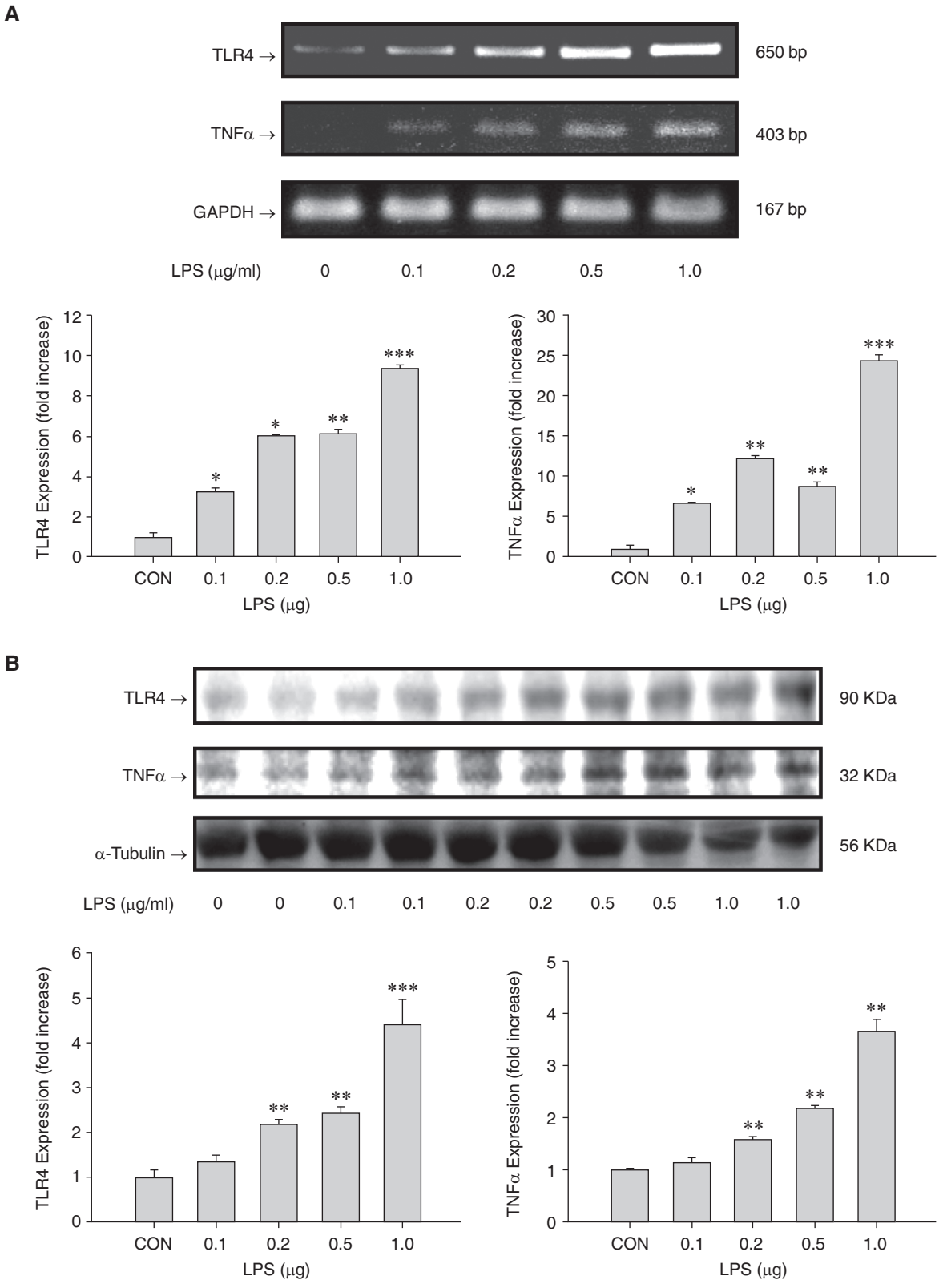

Fig. 2. LPS induces TLR4 mRNA and protein expression in H9c2 cardiomyoblast cells in a time-dependent manner. Tet-On/ERα H9c2 cells were treated with vehicle or LPS (1 μg/ml) for 0-28 h. (A) TLR4 mRNA expression was analyzed by RT-PCR after normalization to GAPDH mRNA. (B) Cell extracts were analyzed by Western blotting with antibodies against proteins as indicated (relative to α-tubulin). Bar graphs show relative intensity of each band which was measured by densitometry. Data represent results from three independent experiments (means ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with unstimulated group).

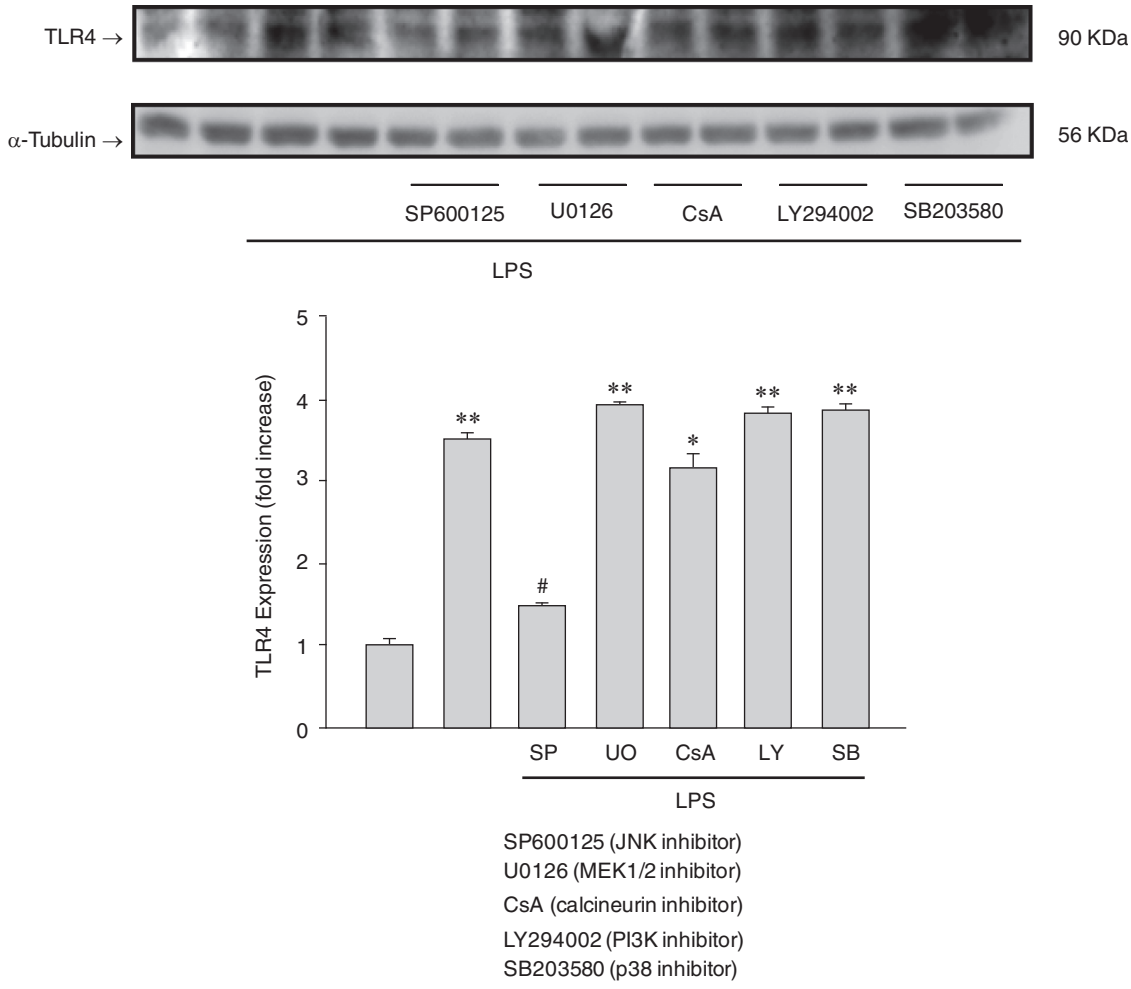

Fig. 3. LPS-induced TLR4 protein expression is mediated through JNK1/2. H9c2 myocardiac cells were pretreated with vehicle, U0126 (ERK1/2 inhibitor, 1 μM), SB203580 (p38 MAPK inhibitor, 1 μM), SP600125 (JNK1/2 inhibitor, 1 μM), CsA (calcineurin inhibitor, 1 μM) or Ly294002 (MEK inhibitor, 1 μM) for 1 h and followed by LPS (1 μg/ml) administration for 24 h. Total proteins of cell extracts were separated by 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against the TLR4 protein. Equal loading was assessed with an anti-α-tubulin antibody. Cells cultured without treatments were used as controls.

2A). LPS also induced TLR4 and TNFα protein expression levels after treatment for 12 h (Fig. 2B). The addition of actinomycin D, a RNA polymerase inhibitor, significantly reduced TLR4 expression in H9c2 cells treated with LPS (Fig. 2C), suggesting that LPS affects TLR4 mRNA expression at the transcription level.

JNK1/2 Mediate LPS-Induced TLR4 Expression in Myocardiac Cells

We further assessed the suppressive effects of inhibitors of U0126, SB203580, SP600125, CsA or Ly294002 on LPS-induced TLR4 expression in myocardial cells. H9c2 cardiomyoblast cells were pretreated with vehicle, U0126 (ERK1/2 inhibitor, 1 μM), SB203580 (p38 MAPK inhibitor, 1 μM), SP600125 (JNK1/2 inhibitor, 1 μM), CsA (calcineurin inhibitor, 1 μM) or Ly294002 (MEK inhibitor, 1 μM) for 1 h prior to the administration of LPS (1 μg/ml) for 24 h, and subsequently subjected to immunoblotting assays. LPS significantly induced TLR4 expression. Pre-treatment with the JNK1/2 inhibitor SP600125 significantly inhibited LPS-induced TLR4 expression. The result suggests that JNK1/2 may mediate LPS-induced TLR4 expression in myocardial cells (Fig. 3).

E_2-Estradiol and Over-Expressed ERα Inhibits TLR4 Expression in LPS-Treated H9c2 Cadiomyoblast Cells

To examine whether E_2-estradiol and over-expressed ERα can inhibit the LPS-induced TLR4 expression, Tet-On/ERα H9c2 cells were treated with E_2 (10^{-8} M), Dox (1 μg/ml), which induces ERα over-

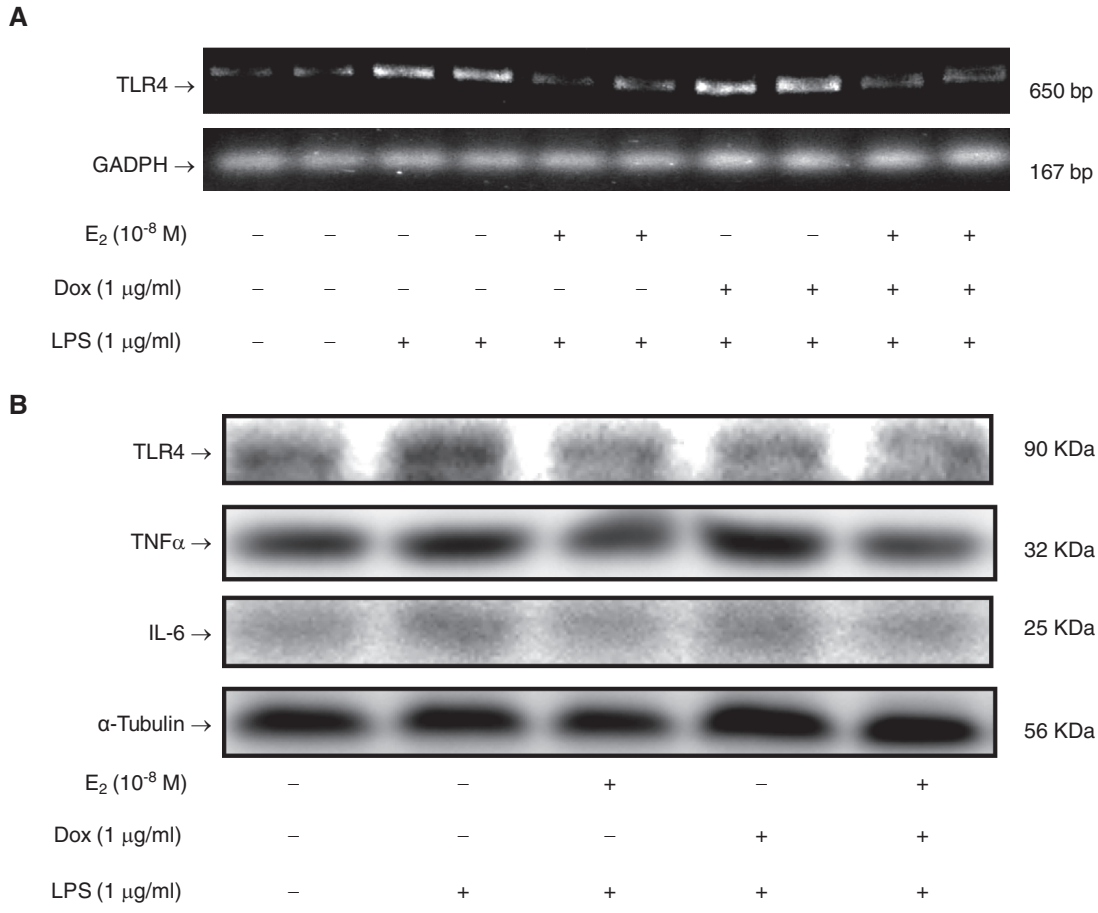

Fig. 4. Estrogen and over-expression of ERα inhibits LPS-induced TLR4 expression. Tet-On/ERα H9c2 cells were incubated with E_2 (10^{-8} M) or Dox (1 μg/ml) in the present of LPS (1 μg/ml) for 24 h. (A) TLR4 mRNA expression was analyzed by RT-PCR after normalization to GAPDH mRNA. (B) Cell extracts were analyzed by Western blotting.

expression, and E_2 (10^{-8} M) plus Dox (1 μg/ml) for 1 h before LPS treatment, E_2 and E_2+ERα over-expression but not ERα over-expression alone significantly inhibited LPS induced TLR4 mRNA expression (Fig. 4A). Interestingly, pretreatment with E_2, ERα over-expression, or both reduced TLR4 protein levels induced by LPS (Fig. 4B). Therefore, ERα appears to be dispensable for the regulation, or there is substantial endogenous ER in H9c2 cells.

LPS Triggers a Distinct Increase in Cytoplasmic HuR, Which Is Significantly Inhibited by E_2, ERα or Both

H9c2 cadiomyoblast cells were treated with vehicle or LPS (1 μg/ml) for 0, 4, 12, 24, and 28 h. HuR was predominantly in the nucleus in un-treatment H9c2 cells. LPS time-dependently caused a significant accumulation of cytoplasmic HuR (Fig. 5A). Pretreatment of Tet-On/ERα H9c2 cells with E_2 (10^{-8} M), Dox (1 μg/ml), and E_2 (10^{-8} M) in the presence of Dox (1 μg/ml) for 1 h before LPS treatment for 24 h, E_2, ERα over-expression, and E_2 plus ERα over-expression reduced HuR protein expression. In contrast, the levels of AUF1 and TTP proteins remained unchanged following LPS, E_2 and/or Dox treatment (Fig. 5B). In Western blot analysis, LPS significantly increased the cytoplasmic level of HuR protein but E_2, ERα over-expression, and E_2 plus ERα over-expression significantly inhibited LPS enhanced cytoplasmic HuR expression, and the level of nuclear HuR did not decrease concomitantly with the increase in cytoplasmic HuR (Fig. 5C). Therefore, the Tet-On/ER system is not tight enough to prevent leakage of ER expression.

Discussion

In this study, we found that MAPK signaling pathways play critical roles in LPS enhanced TLR4 expression and stimulates an inflammatory response in H9c2 cardiomyoblast cells. LPS enhanced MAPK signaling transduction and activated HuR production to transcriptionally promote TLR4 gene expression.

LPS from gram-negative bacteria is considered

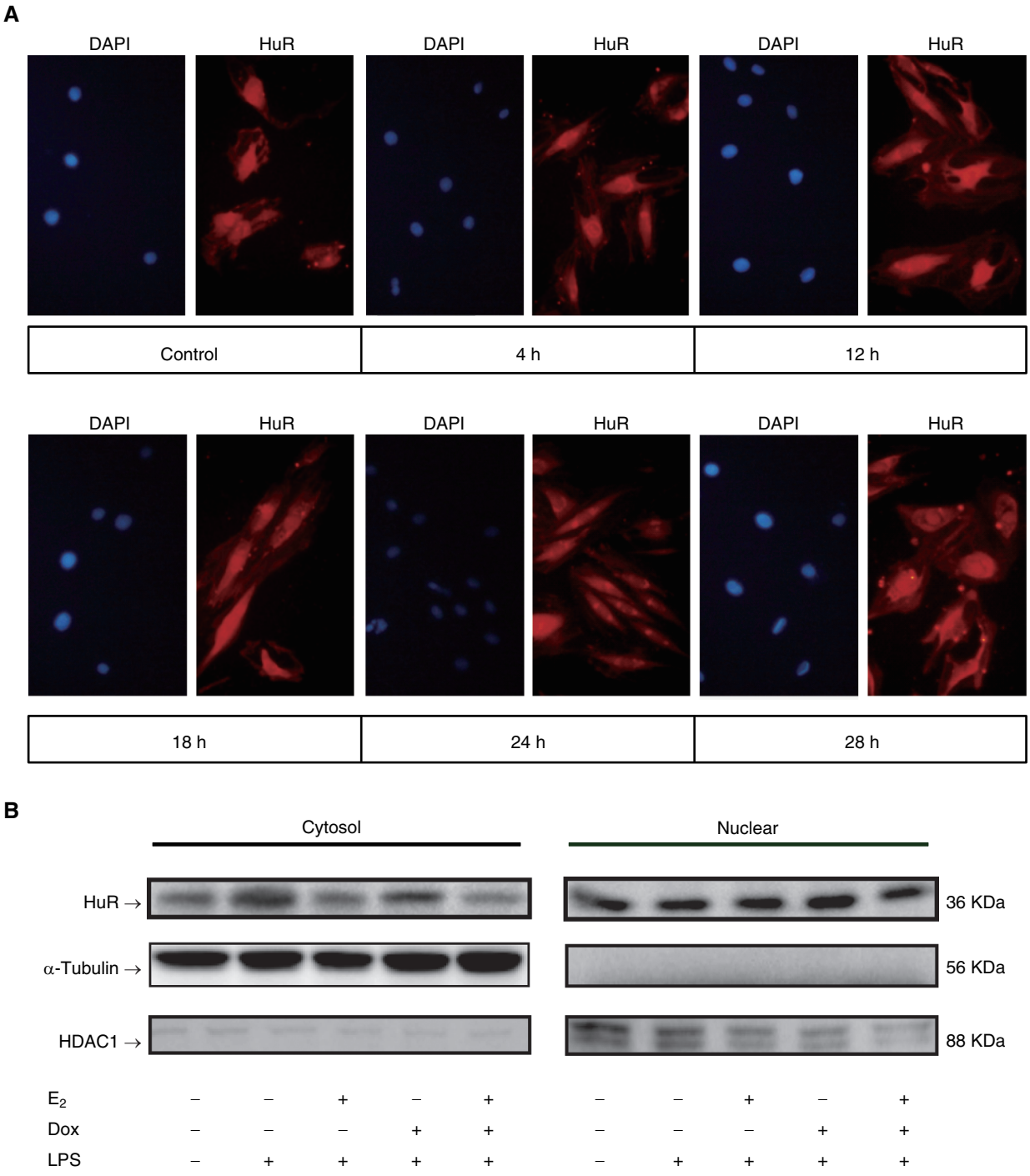

Fig. 5. E_2 and ERα over-expression reverse the LPS-induced HuR nuclear export. (A) Tet-On/ERα H9c2 cells were incubated with LPS (1 μg/ml) for 0-28 h. Cells were then fixed, and the immunoflurescence staining with an antibody against HuR was performed and visualized with a fluorescence microscope coupled with an image analysis system. (B) Tet-On/ERα H9c2 cells were incubated with E_2 (10^{-8} M), Dox (1 μg/ml) in the presence of LPS (1 μg/ml) for 24 h. Cell lysates and nuclear extracts were prepared, and the levels of cytoplasmic HuR were determined by Western blotting.

to be a strong stimulator of the pathogenesis of cardiac disease (18). Evidences suggest that plasma concentrations of LPS rise in patients with chronic heart failure (CHF) and activated immune system (29). The present study shows that cardiomyocytes exhibit lower expression of TLR4 under basal conditions. Low concentrations of LPS may contribute to the increased severity of ischemic heart disease (24). Furthermore, studies have demonstrated that LPS directly decreases contractility (2) and dramatically induces TNFα expres-

sion in cardiomyocytes through binding to TLR4 (3). Clinical studies have shown that severe cardiac contractile dysfunction is the common symptoms in patients with sepsis (7).

The existence of TLR4 in myocardial cells may be a fundamentally significant contribution for the crucial pathological relationship between inflammation and cardiovascular disorders (41). TLR4 expression under LPS stimulation is controlled by transcriptional and posttranscriptional mechanisms, which may be enhanced by MAPK signaling pathways (26). The MAPK signaling pathways play an important role in signal transduction in eukaryotic cells that transduce signals following growth or stress stimulation. MAPK proteins, including.

It has been well known that p38, JNK/SAPK, and ERK1/2 are associated with inflammatory stimuli and oxidative stress. The activation of MAPK has been showed to participate in cardiac pathologies (4). In this study, we found that incubation of LPS treated myocardial cells with JNK1/2 inhibitor SP600125 resulted in significant inhibition of LPS-induced TLR4 protein expression, suggesting that JNK1/2 may be a key mediator when LPS binds to TLR4.

There are two different forms of the ER, ERα and ERβ. Each ER is encoded by a separate gene and differs in structure, tissue location, and functions (13). The similar activity of AF2 in ERα and ERβ shows similar ability in to bind coactivators (14). To compare the activity of AF1 in ERα and E_2 and/or ERβ, the two ERs were examined with different ligands in estrogen receptor element (ERE). The ability of ERE or SP-1 binding in ERα is better than ERβ, but ERβ has a higher affinity to AP-1 (31).

Studies have shown that women have lower mortality rate of sepsis or related multi-organ diseases than men (34). Women have lower myocardial inflammatory responses, lower levels of cytokine production and better myocardial function after burn trauma injury (17, 27, 37). In addition, clinical research shows that premenopausal women have lower TNFα product compared with men or postmenopausal women. In the present study, we observed that administration of E_2, and/or Dox, which induces ERα overexpression, significantly provided cardioprotective effects by repressing LPS-induced TLR4 expression and down-regulated proinflammatory TNFα production.

Our results show that LPS-induced TLR4 expression was blocked by actinomycin D, an RNA polymerase inhibitor, suggesting that regulation of TLR4 expression by LPS might be mediated at the transcriptional level. The basal expression of proteins associated with inflammatory responses is potentially unstable in normal cells, possibly because of facile mRNA degradation. Unstable mRNAs often contain AREs in their 3'UTR (38). HuR binds strongly to AREs and stabilizes mRNA. LPS markedly increased the cytoplasmic level of HuR. With pretreatment of Tet-On/ERα H9c2 cells, E_2, ERα over-expression, and E_2 plus ERα over-expression reduced HuR protein expression and reversed LPS-induced translocation of HuR from cell nucleus to cytoplasm. However, the nuclear level of HuR was abundant and did not change. ERα appears to be dispensable for the above regulation, or there is substantial endogenous ER in H9c2 cells, therefore, there was no synergistic effect caused by E_2 plus ERα. In addition, there could be some form of feedback mechanism that adjusted the effects of E_2 and/or ERα to generate the correct cellular response.

In conclusion, we found that LPS-enhanced TLR4 mRNA was mediated by HuR expression and up-regulated the expression of TLR4 through JNK1/2 pathway in myocardial cells. In addition, using Dox-induced Tet-On ERα H9c2 myocardial cells, E_2 and/or ERα significantly abolished the LPS-induced cytoplasmic HuR protein level to reduce TLR4 mRNA stability.

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