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RESEARCH PAPER

Regulation of cyclooxygenase-2 and cytosolic phospholipase A_2 gene expression by lipopolysaccharide through the RNA-binding protein HuR: involvement of NADPH oxidase, reactive oxygen species and mitogen-activated protein kinases

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BACKGROUND AND PURPOSE

Lipopolysaccharide (LPS)-induced expression of cyclooxygenase-2 (COX-2) and cytosolic phospholipase A_2 ($cPLA_2$) has been implicated in several respiratory diseases. HuR is known to enhance the expression of genes by binding to 3′-untranslated region (3′-UTR) of mRNA and stabilizing mRNA. However, the exact mechanisms by which HuR affects the stability of mRNA and modulates LPS-induced COX-2 and $cPLA_2$ expression in human tracheal smooth muscle cells (HTSMCs) are not known.

EXPERIMENTAL APPROACH

The expression of prostaglandin E_2 (PGE_2) was measured by ELISA, and pro-inflammatory proteins were determined by use of a promoter assay, PCR or Western blot analysis. Overexpression of siRNAs to knock down the target components was used to manipulate the expression of HuR. Release of reactive oxygen species (ROS) was detected by fluorescence dye. The activation of signalling components was assessed by comparing phosphorylation levels, localization of protein kinases or coimmunoprecipitation assay.

KEY RESULTS

LPS induced COX-2 and $cPLA_2$ expression via post-translational regulation of mRNA stabilization, which were attenuated by transfection with HuR siRNA in HTSMCs. In addition, LPS-stimulated NADPH oxidase activation and ROS generation were attenuated by the NADPH oxidase inhibitors diphenyleneiodonium chloride (DPI) and apocynin (APO). Generation of ROS induced phosphorylation of p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK and JNK1/2, which was attenuated by DPI and APO and the ROS scavenger N-acetylcysteine.

CONCLUSIONS AND IMPLICATIONS

These results suggested that in HTSMCs, LPS-induced COX-2 and $cPLA_2$ expression is mediated through NADPH oxidase/ROS-dependent MAPKs associated with HuR accumulation in the cytoplasm. Activated MAPKs may regulate the nucleocytoplasmic shuttling of HuR, and thus induce the cytoplasmic accumulation of HuR.

Abbreviations

AA, arachidonic acid; AMPK, AMP-activated kinase; APO, apocynin; BCA, bicinchoninic acid; COPD, obstructive pulmonary disease; COX-2, cyclooxygenase-2; $cPLA_2$, cytosolic phospholipase A_2; DPI, diphenyleneiodonium chloride; ECL, enhanced chemiluminescence; ECM, extracellular matrix; HNS, HuR nucleocytoplasmic shuttling sequence; HTSMCs, human tracheal smooth muscle cells; HuR, Hu antigen R; LPS, lipopolysaccharide; LTs, leukotrienes; NAC, N-acetylcysteine; PGs, prostaglandins; RRM, RNA-recognition motifs

Introduction

Human tracheal smooth muscle cells (HTSMCs) are thought to be end-response effector cells that regulate regional differences in ventilation by contracting in response to various neurotransmitters, pro-inflammatory mediators and exogenous substances released under homeostatic or pathological conditions such as asthma (Hirst *et al.*, 2004). On the other hand, HTSMCs are also important sources of pro-inflammatory cytokines, chemokines and growth factors and extracellular matrix (ECM) components (Lazaar and Panettieri, 2006), which contribute to the development and perpetuation of airway inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Johnson *et al.*, 2001). Cytosolic phospholipase A_2 ($cPLA_2$) and cyclooxygenase-2 (COX-2) are pro-inflammatory proteins; $cPLA_2$ catalyses the hydrolysis of the *sn*-2 position of membrane glycerophospholipids, leading to produce lysophospholipids and free fatty acids including arachidonic acid (AA) (Kudo and Murakami, 2002), which in turn are converted by COX-2 to form various bioactive lipophilic compounds called eicosanoids, such as prostaglandins (PGs) (Samad *et al.*, 2002). The characters of $cPLA_2$ and COX-2 in inflammation have been proved in several studies. In $cPLA_2$-null mice, pulmonary edema, neutrophil sequestration and deterioration of gas exchange induced by lipopolysaccharide (LPS), zymosan administration and acid aspiration are markedly reduced (Nagase *et al.*, 2000). Enhanced levels of COX-2-related prostaglandin E_2 (PGE_2) have been found to correlate with the severity of airflow limitation in COPD (Chen *et al.*, 2008). Thus, the inhibition of $cPLA_2$-COX-2 related mechanisms may also provide a therapeutic approach for acute lung injury.

Allergen exposure is important in host allergic sensitization and as a common precipitant of asthmatic symptoms in both children and adults (Lemanske and Busse, 2006). Bacterial LPS, present in allergic dust, seems to be one of major candidates for the induction of inflammatory reaction (Thorn, 2001; Schroder and Arditi, 2007). LPS, a key component of outer membranes of gram-negative bacteria, is composed of three structural elements including a core oligosaccharide, an O-specific chain made up of repeating sequences of LPS and a lipid A component, which is responsible for the pro-inflammatory properties of LPS (Miller *et al.*, 2005). In previous studies, we have demonstrated that stimulation with LPS leads to the infiltration of leucocytes into the lung via transcriptional up-regulated expression of adhesion molecules (Lin *et al.*, 2009). However, the exact mechanisms underlying LPS-induced expression of $cPLA_2$ and COX-2 in HTSMCs have not been completely elucidated.

Gene regulation can occur at the level of proteins (selective degradation), DNA (differential transcription) and RNA (mRNA stability) (Brennan and Steitz, 2001). HuR (Hu antigen R; ELAVL1) is a member of the embryonic lethal, abnormal vision, Drosophila-like ELAV family of RNA-binding proteins and is ubiquitously expressed. HuR is involved in a variety of physiological and pathological processes, including cell growth, differentiation and inflammation (Abdelmohsen *et al.*, 2007). HuR contains three RNA-recognition motifs (RRM) through which it binds to adenylate- and uridylate (AU)-rich elements (AREs)-containing mRNAs and induces a marked increase in the half-life of many short-lived mRNAs, possibly by antagonizing the recruitment of competitive mRNA-destabilizing factors. As HuR is mainly located within the nucleus, cytosolic export is an important prerequisite for its protective effects on mRNAs and this is regulated by an HuR nucleocytoplasmic shuttling sequence (HNS) on its structure (Fan and Steitz, 1998). The nuclear export of HuR proteins is also regulated by various stimuli through several intracellular signalling pathways including MAPKs (Subbaramaiah *et al.*, 2003), AMP-activated kinase (AMPK) (Wang *et al.*, 2002), and different members of the PKC family (Doller *et al.*, 2007; 2008a). Therefore, we investigated whether HuR plays a key role in the regulation of LPS-induced expression of $cPLA_2$ and COX-2 in HTSMCs.

HuR has been found to stabilize COX-2 mRNA in human mesangial cells, ovarian cancer cells and human keratinocytes exposed to various stimuli (Doller *et al.*, 2008a; Oyesanya *et al.* 2008; Zhang and Bowden, 2008). Anti-rheumatic drugs, such as aurothiomalate, reduce the expression of COX-2 and PGE_2 production in chondrocyte cultures and in human cartilage by enhancing the degradation of COX-2 mRNA through mechanisms involving reduced HuR expression (Nieminen *et al.*, 2008). In contrast, little is known about the stabilization of $cPLA_2$ mRNA, although the existence of putative HuR-binding sites on 3′-UTR of human $cPLA_2$ mRNA has been suggested (Tsou *et al.*, 2008). Thus, whether LPS stimulates $cPLA_2$ or COX-2 expression in HTSMCs through mechanisms involving HuR-mediated stabilization of mRNA expression needs to be determined.

Several studies have also revealed that stimulation of LPS may lead to reactive oxygen species (ROS) production in human umbilical vein endothelial cells and macrophage (Al Laham *et al.*, 2010; Kong *et al.* 2010). Generation of ROS may result from the activation of NADPH oxidase in response to various extracellular stimuli (Frey *et al.*, 2009). The structure of NADPH oxidase including the catalytic centre of membrane-integrated protein $gp91^{phox}$, which tightly complexed with $p22^{phox}$, and the activation-related association components of $p47^{phox}$, $p67^{phox}$ and the small GTPase Rac, which normally reside in the cytoplasm (Sumimoto *et al.*, 2005). However, whether NADPH oxidase-dependent generation of ROS affects HuR-mediated COX-2 and $cPLA_2$ expression in LPS-stimulated HTSMCs remains to be defined. Here we report that LPS-induced COX-2 and $cPLA_2$ expression is mediated through manipulation of the HuR level in HTSMCs. We also provide evidence that LPS regulates the physical interaction between HuR and activated MAPKs, leading to the nucleocytoplasmic shuttling of HuR via a mechanism dependent on NADPH oxidase, ROS and MAPKs.

Methods

Materials

Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12 medium) fetal bovine serum (FBS), and TRIZOL were purchased from Invitrogen (Carlsbad, CA, USA). Hybond C membrane, enhanced chemiluminescence (ECL)

Western blotting detection system and Hyperfilms were from GE Healthcare Biosciences (Buckinghamshire, UK). Monoclonal antibodies – cPLA$_2$, COX-2, HuR, p47^{phox}, p67^{phox}, β-actin – and polyclonal antibodies – p44 MAPK, p42 MAPK, p38 MAPK, JNK2 – were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PhosphoPlus p42/p44 MAPK, p38 MAPK and SAPK/JNK antibody kits were from Cell Signalings (Danver, MA, USA). Anti-GAPDH antibody was from Biogenesis (Boumemouth, UK). N-acetylcysteine (NAC), diphenyleneiodonium chloride (DPI), apocynin (APO), U0126, SB202190 and SP600125 were from Biomol (Plymouth Meeting, PA, USA). Bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL, USA). LPS, enzymes and other chemicals were from Sigma (St. Louis, MO, USA). The nomenclatures of all drugs and molecular targets conform to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2009).

Cell culture

HTSMCs were purchased from ScienCell Research Lab (San Diego, CA, USA) and cultured as previously described (Lin *et al.*, 2001). When the cultures reached confluence, cells were treated with 0.05% (w v^{-1}) trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) for 5 min at 37°C. The cell suspension was diluted with DMEM/F-12 containing 10% FBS to a concentration of 2×10^5 cells mL^{-1}. The cell suspension was plated onto (1 mL per well) 12 well culture plates (2 mL per well) 6 well culture plates and (10 mL per dish) 10 cm culture dishes for the measurement of protein expression and mRNA accumulation. Experiments were performed with cells from passages 3 to 8.

Total RNA extraction and gene expression analysis

Total RNA was extracted from HTSMCs using Trizol, as previously described (Lin *et al.*, 2001). The cDNA containing 0.5 μg RNA was used as templates to analyse specific gene mRNA level. Oligonucleotide primers for β-actin, cPLA$_2$ and COX-2 were as follows: for β-actin: 5′-TGACGGGGTCA CCCACACTGTGCCCATCTA-3′ (Sense), 5′-CTAGAAGCATTT GCGGTGGACGATG-3′ (Anti-sense); for cPLA$_2$: 5′-CTC-AC ACCACAGAAAGTTAAAAGAT-3′ (Sense), 5′-GCTACCACAGG CACATCA-CG-3′ (Anti-sense); for COX-2: 5′-TTCAAATGAG ATTGTGGGAAAATTGCT-3′ (Sense), 5′-AGATCATCTCTGCC TGAGTATCTT-3′ (Anti-sense). The amplification profile includes one cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C for 1 min, extension at 72°C for 1 min, and then one cycle of final extension at 72°C for 5 min. The expression of β-actin was used as an internal control for the assay of a constitutively expressed gene. Real-time PCR was performed with a TaqMan gene expression assay system (Applied Biosystems, Carlsbad, CA, USA), using primer and probe mixtures for COX-2, cPLA$_2$ and GAPDH as endogenous control. The primer and probe sequences for gene amplification were COX-2 (Cat. no. Hs01573471_m1), cPLA$_2$ (Cat. no. Hs00996915_m1) and GAPDH (Cat. no. Hs99999905_m1). The reactions were performed in a StepOnePlus real-time PCR system (Applied Biosystems) under the following thermal cycling conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The threshold cycle value was normalized to that of GAPDH. Relative gene expression was determined by the ΔΔCt method, where Ct meant threshold cycle. All experiments were performed in triplicate.

Preparation of cell extracts and Western blot analysis

After incubation with LPS, the cells were then rapidly washed with ice-cold phosphate-buffered saline (PBS), scraped and collected by centrifugation at 1000× *g* for 10 min. The collected cells were lysed with an ice-cold lysis buffer. The lysates were centrifuged at 45 000× *g* for 1 h at 4°C to yield the whole cell extract. Samples from these supernatant fractions (30 μg protein) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% running gel. Proteins were transferred to nitrocellulose membrane and then incubated successively at room temperature with 5% bovine serum albumin (BSA) in TTBS for 1 h. Membranes were incubated overnight at 4°C with an anti-cPLA$_2$, COX-2, HuR, β-actin, phospho-p42/p44 MAPK, phospho-p38 MAPK or phospho-JNK1/2 antibody. Membranes were incubated with a 1:2000 dilution of anti-rabbit or anti-mouse horseradish peroxidase Ab for 1 h. The immunoreactive bands were detected by ECL reagents (PerkinElmer, Waltham, MA, USA).

Cell fraction isolation

After stimulation with LPS, the membrane, cytosolic and nuclear fractions were prepared by centrifugation. Samples from various fractions (30 μg protein) were denatured and subjected to SDS-PAGE using a 10% running gel. Proteins were transferred to nitrocellulose membrane and then incubated successively at room temperature with 5% BSA in TTBS for 1 h. The translocation of HuR, p47^{phox} or p67^{phox} was analysed by Western blot using an anti-HuR, p47^{phox}, p67^{phox} or anti-β-actin (as an internal control) polyclonal antibody. The immunoreactive bands were detected by ECL reagents (PerkinElmer).

Immunofluorescence staining

HTSMCs were plated on 6 well culture plates with coverslips. Cells were treated with LPS for the indicated time intervals, washed twice with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 30 min, and then permeabilized with 0.3% Triton X-100 in PBS for 15 min. The staining was performed by incubating with 10% normal goat serum in PBS for 30 min, followed by incubating with an anti-HuR monoclonal antibody for 1 h in PBS with 1% BSA, washing three times with PBS, incubating for 1 h with FITC-conjugated goat anti-rabbit antibody in PBS with 1% BSA, washing three times with PBS, and finally mounting with aqueous mounting medium. The images were observed under a fluorescence microscope (Axiovert 200M, Carl Zeiss light Microscopy, Gottingen, Germany).

Luciferase activity assay

The COX-2-luciferase plasmids were constructed with a region spanning –459 to +9 bp of COX-2 promoter into pGL3-basic vector. The cPLA$_2$-luciferase plasmids were con-

structed with a region spanning –595 to +75 bp of $cPLA_2$ promoter into a pGL3-basic vector. The plasmids were prepared by using QIAGEN plasmid DNA preparation kits. COX-2-luciferase or $cPLA_2$-luciferase plasmid was transfected into HTSMCs using Genejammer transfection reagent (Stratagene, La Jolla, CA, USA) according to the instructions of the manufacturer. To assess promoter activity, cells were collected and disrupted by sonication in a lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100 and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using a luciferase assay system (Promega, Madison, WI, USA) according to the instructions of the manufacturer. Firefly luciferase activities were normalized to β-galactosidase activity.

Overexpression of HuR plasmid or HuR siRNA

To generate the mammalian expressed HuR plasmid, human HuR cDNA was PCR-amplified using the following pair of oligonucleotides: 5′-CGGGAATTCATACAATGTCTAATGGTT ATGAAGACC (sense) and 5′-CGGTCTAGAGAGCGTTATTT GTGGGACTTG (antisense); the PCR fragment was then digested with restriction enzymes *Eco*RI and *Xba*I, and cloned into the eukaryotic expression vector pCDNA3 (Invitrogen) as described by Fan and Steitz (1998). ON-TARGETplus siRNA of HuR (Accession no. NM_001419) was purchased from Dharmacon Research (Lafayette, CO, USA).

HTSMCs were plated at 3×10^5 cells mL^{-1} (1 mL per well) in 12 well culture plates for 24 h, reaching about 80% confluence, and 0.4 mL of DMEM/F-12 containing 10% FBS was added to each well. The DNA Metafectene reagent complex was prepared according to the instructions of the manufacturer (Biontex, Martinsried/Planegg, Germany). The amount of transfected DNA was kept constant with 1 μg of HuR plasmid or with 100 nM of siRNA for each well. The DNA Metafectene complex (0.1 mL) was added to each well and then incubated at 37°C for 24 h. The cells were washed twice with PBS and maintained in DMEM/F-12 containing 1% FBS for 24 h (transfection with HuR plasmid or pcDNA3) or 72 h (transfection with siRNA) before treatment with LPS for the indicated time intervals.

ROS measurement by 7′-dichlorofluorescein-diacetate (DCF-DA) fluorescence

Formation of ROS in HTSMCs was determined by the DCF-DA fluorescence method. HTSMCs (~90% confluence in 12 well plates) were loaded with 1 μM DCF-DA for 30 min in phenol red-free medium at 37°C in a 95% air–5% CO_2 environment. The medium containing DCF-DA was aspirated, and the cells were washed twice with PBS, and replenished with 0.5 mL of phenol red- and serum-free RPMI medium. Some cells were pretreated with the inhibitors for 1 h followed by exposure to LPS (100 μg·mL^{-1}) for the indicated time intervals. The cells were washed twice with ice-cold PBS and scraped using lysis buffer (1X PBS containing 20% alcohol and 0.1% Tween 20). The cell lysates were transferred to 1.5-mL Eppendorf vials and centrifuged at 10 000× *g* for 1 min at 4°C. Fluorescence of oxidized DCF-DA in cell lysates, an index of formation of ROS, was measured by a fluorimeter (Appliskan^R, Thermo, Vantaa, Finland) with excitation and emission set at 490 and 530 nm respectively.

Determination of NADPH oxidase activity

Cells were grown on 6 well culture plates and incubated with LPS for the indicated time intervals. Cells were gently scraped and centrifuged at 400× *g* for 10 min at 4°C. The cell pellet was resuspended in 35 μL of ice-cold RPMI 1640 medium per vial and then was kept on ice. Five microliters of the cell suspension (0.2×10^5 cells) were added to a final 200 μL volume of pre-warmed (37°C) RPMI 1640 medium containing either NADPH (1 μM) or lucigenin (20 μM), to initiate the reaction, followed by immediate measurement of chemiluminescence using a luminometer (Appliskan, Thermo) in out-of-coincidence mode. Appropriate blanks and controls were established, and chemiluminescence was recorded.

Coimmunoprecipitation assay

Cell lysates containing 1 mg of protein were incubated with 2 μg of anti-HuR, anti-p42/p44 MAPK, anti-p38 MAPK or anti-JNK1/2 Ab at 4°C for 1 h, and then 10 μL of 50% protein A-agarose beads was added and mixed for 16 h at 4°C. The immunoprecipitates were collected and washed three times with lysis buffer without Triton X-100, 5X Laemmli buffer was added, and then subjected to Western blot analysis using an anti-HuR, p42/p44 MAPK, p38 MAPK or JNK1/2 Ab.

Measurement of PGE_2 generation

HTSMCs were cultured on 6 well culture plates. The cells were incubated with LPS (100 μg mL^{-1}) for the indicated time intervals at 37°C. The medium was collected and stored at –80°C. PGE_2 was assayed using a PGE_2 enzyme immunoassay kit (Cayman, Ann Arbor, MI, USA) according to the instructions of the manufacturer.

Statistical analysis of data

All data are expressed as the mean ± standard error of the mean using the GraphPad Prism Program (GraphPad, San Diego, CA, USA). Quantitative data were analysed by one-way analysis of variance followed by Tukey's *post hoc* test. A value of $P < 0.05$ was considered significant.

Results

LPS induces de novo $cPLA_2$ and COX-2 gene expression and PGE_2 synthesis

LPS functioning as a major pro-inflammatory factor has been shown to induce airway inflammation. Here we determined whether COX-2 and $cPLA_2$ expression were up-regulated by LPS. Serum-starved HTSMCs deprived of serum were incubated with 100 μg·mL^{-1} LPS for various time intervals. As shown in Figure 1A, LPS induced $cPLA_2$ and COX-2 protein expression in a time-dependent manner. LPS-induced $cPLA_2$ expression was observed to be significant at 6 h, reached a peak within 16 h, and lasted up to 24 h. In addition, LPS-induced COX-2 expression was significant within 2 h, reached a maximum within 8 h, and thereafter declined close

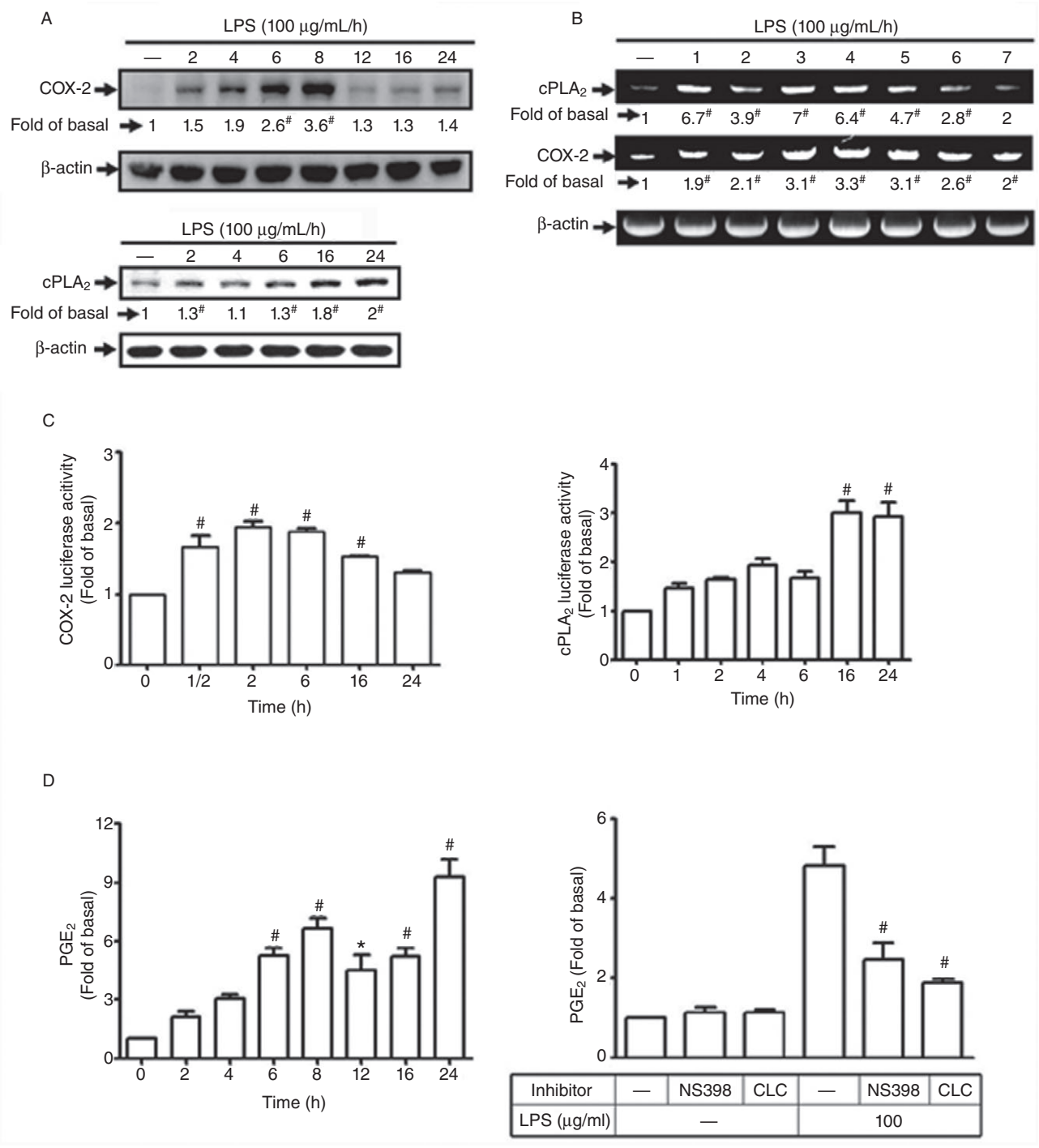

Figure 1

Lipopolysaccharide (LPS) induced cyclooxygenase-2 (COX-2) and cytosolic phospholipase A_2 (cPLA$_2$) expression and prostaglandin E_2 (PGE$_2$) synthesis in human tracheal smooth muscle cells (HTSMCs). (A) HTSMCs were incubated with 100 μg·mL^{-1} of LPS for the indicated time intervals. COX-2 and cPLA$_2$ protein expression were determined by Western blot analysis. (B) For mRNA expression, HTSMCs were incubated with LPS (100 μg·mL^{-1}) for the indicated time intervals. mRNA was extracted and analysed by RT-PCR. (C) HTSMCs were transiently transfected with either cPLA$_2$-luc or COX-2-luc reporter gene together with β-galactosidase plasmid and then incubated with 100 μg·mL^{-1} of LPS for the indicated time intervals. Luciferase activity was determined and normalized with β-galactosidase activity. (D) HTSMCs were pretreated without or with NS-398 (10 μM) or celecoxib (CLC; 10 μM) for 1 h and then incubated with LPS for the indicated time intervals or 6 h respectively. The culture supernatants were collected to measure PGE$_2$ concentration. The basal level of PGE$_2$ was approximately 256 pg·mL^{-1}. Data are expressed as mean ± SEM of five independent experiments ($n = 5$). *$P < 0.05$, #$P < 0.01$ as compared with the cells exposed to vehicle alone.

to the basal level. The blot was stripped and re-probed with an anti-β-actin antibody to demonstrate equivalent amounts of GAPDH expression.

To further examine whether the effects of LPS on $cPLA_2$ and COX-2 expression occurred at the level of transcription, $cPLA_2$ and COX-2 mRNA expression were determined by use of RT-PCR. As shown in Figure 1B, LPS induced $cPLA_2$ or COX-2 mRNA accumulation in a time-dependent manner. The maximal response of $cPLA_2$ mRNA was obtained within 3 h and lasted up to 6 h during the period of observation. The maximal response of COX-2 mRNA occurred within 3 to 6 h after stimulation with LPS. Moreover, the promoter activities of $cPLA_2$ and COX-2 were also enhanced by LPS stimulation, with maximal responses within 16 h and 2 h respectively (Figure 1C). The newly synthesized $cPLA_2$ and COX-2 were biologically functional, indicated by the increased release of PGE_2 in response to LPS (Figure 1D). The synthesis of PGE_2 induced by LPS was found to be biphasic with the first phase maximum occurring within 8 h and the second maximal response within 24 h. LPS-induced PGE_2 synthesis was attenuated by the COX-2 inhibitors, NS-398 and celecoxib (Figure 1D), suggesting that the release of PGE_2 was mediated through COX-2 expression. These data suggest that LPS-stimulated PGE_2 synthesis is mediated through up-regulation of $cPLA_2$ and COX-2 expression in HTSMCs.

LPS induces $cPLA_2$ and COX-2 expression via the generation of ROS

To investigate whether ROS participates in LPS-induced $cPLA_2$ and COX-2 expression, HTSMCs were pretreated with various concentrations of an ROS scavenger (NAC) for 1 h and then incubated with 100 $\mu g \cdot mL^{-1}$ of LPS for 24 h. Cell lysates were analysed by Western blot using an anti-$cPLA_2$ or anti-COX-2 Ab. We found that LPS-induced expression of $cPLA_2$ and COX-2 proteins was attenuated by pretreatment with NAC in a concentration-dependent manner (Figure 2A). LPS-induced expression of $cPLA_2$ and COX-2 mRNA was also reduced by pretreatment with NAC (Figure 2B), revealing the involvement of ROS generation in LPS-induced $cPLA_2$ and COX-2 expression. Moreover, the levels of ROS production were determined by using DCF-DA. LPS-stimulated accumulation of intracellular ROS was significant within 15 min and lasted up to 3 h (Figure 2C). Pretreatment with NAC significantly attenuated LPS-induced ROS generation (Figure 2D). Taken together, these data suggest that ROS generation contributes to the expression of $cPLA_2$ and COX-2 in response to LPS in HTSMCs.

Participation of NADPH oxidase in LPS-induced $cPLA_2$ and COX-2 expression

Several lines of evidence have demonstrated that ROS contribute to the expression of inflammatory proteins in various cell types (Bedard and Krause, 2007). NADPH oxidase is an important enzymatic source for the production of ROS under various pathological conditions. Thus, the role of NADPH oxidase in ROS generation associated with $cPLA_2$ and COX-2 expression was investigated in HTSMCs challenged with LPS. As shown in Figure 3A, pretreatment of HTSMCs with inhibitors of NADPH oxidase (DPI and APO) significantly attenuated LPS-induced $cPLA_2$ and COX-2 protein expression. Moreover, pretreatment with either DPI or APO also inhibited $cPLA_2$ and COX-2 mRNA expression induced by LPS (Figure 3B). These results suggest that NADPH oxidase plays a key role in LPS-induced $cPLA_2$ and COX-2 expression in HTSMCs.

The involvement of NADPH oxidase in these responses may be due to the generation of ROS. As shown in Figure 3C, LPS-induced ROS generation was reduced by pretreatment with inhibitors of NADPH oxidase (DPI and APO). Generation of intracellular ROS may result from the activation of NADPH oxidase, which is composed of $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, p22 and gp91. It has been demonstrated that $p47^{phox}$ organizes the translocation of other cytosolic factors, hence its designation as 'organizer subunit' (Barbieri *et al.*, 2004). We found that LPS induced a significant increase in translocation of $p67^{phox}$ and $p47^{phox}$ from the cytosol to the membrane (Figure 3D) and these effects were attenuated by pretreatment with DPI or APO (Figure 3E). Moreover, the enhanced activity of NADPH oxidase induced by LPS was significantly attenuated by pretreatment with DPI or APO (Figure 3F). These results indicate that the activation of NADPH oxidase and generation of ROS have critical roles in LPS-induced $cPLA_2$ and COX-2 expression in HTSMCs.

LPS-induced $cPLA_2$ and COX-2 expression via NADPH oxidase/ROS-dependent MAPKs cascade

To investigate whether p42/p44 MAPK, p38 MAPK or JNK1/2 participates in LPS-induced $cPLA_2$ and COX-2 expression, cells were pretreated with inhibitors of MEK1/2 (PD98059 and U0126), p38 MAPK (SB20190) or JNK (SP600125) for 1 h and then incubated with 100 $\mu g \cdot mL^{-1}$ of LPS for 24 h or 6 h. As shown in Figure 4A and B, pretreatment with PD98059, U0126, SB202190 or SP600125 inhibited LPS-induced $cPLA_2$ or COX-2 protein and mRNA expression in a concentration-dependent manner. Further LPS-stimulated phosphorylation of MAPKs was shown to be mediated by NADPH oxidase-dependent generation of ROS, as pretreatment with NAC, DPI or APO attenuated LPS-induced phosphorylation of p42/p44 MAPK, p38 MAPK and JNK1/2 (Figure 4C). Collectively, these results reveal that in HTSMCs, LPS-induced $cPLA_2$ and COX-2 expression is mediated through an NADPH oxidase/ROS-dependent activation of MAPKs cascade.

LPS stimulates nucleocytoplasmic shuttling of HuR in HTSMCs

Several studies have suggested the involvement of HuR in mRNA stabilization of inflammatory genes. We investigated whether HuR participates in the process of $cPLA_2$ or COX-2 expression in LPS-stimulated HTSMCs. HTSMCs were transiently transfected with HuR siRNA and then incubated with 100 $\mu g \cdot mL^{-1}$ LPS for 24 or 6 h. Cell lysates were subjected to Western blot analysis using an anti-$cPLA_2$ or anti-COX-2 antibody. Transfection with HuR siRNA significantly reduced HuR expression and also attenuated LPS-induced expression of $cPLA_2$ and COX-2 (Figure 5A), suggesting that HuR is involved in $cPLA_2$ and COX-2 expression in HTSMCs. The cytosolic export of HuR needs to occur for it to become biologically active and involved in gene transcriptional regulation, hence, cytosolic and nuclear fractions were prepared

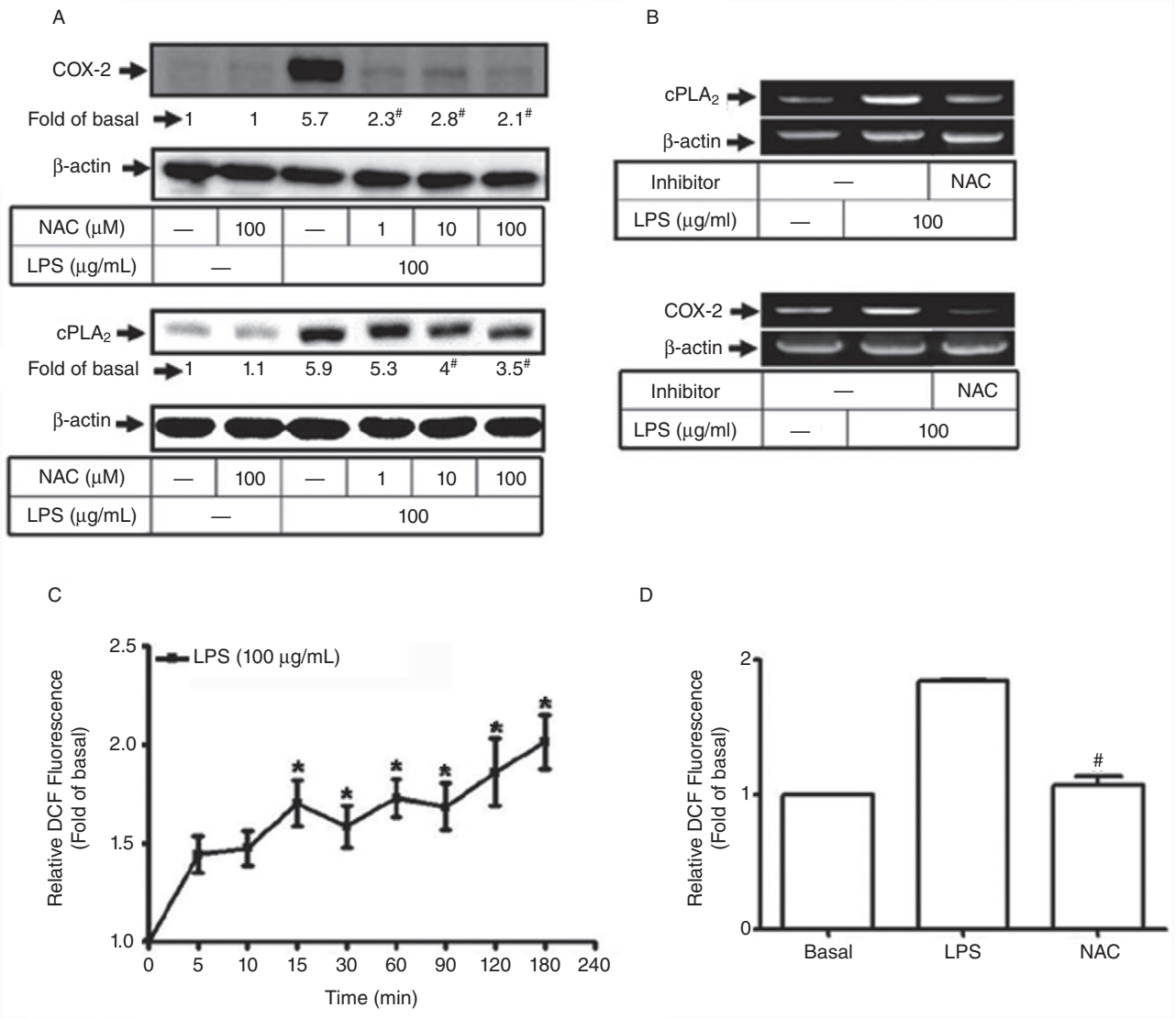

Figure 2

Involvement of reactive oxygen species (ROS) in lipopolysaccharide (LPS)-mediated cyclooxygenase-2 (COX-2) and cytosolic phospholipase A_2 (cPLA$_2$) expression. (A) Human tracheal smooth muscle cells (HTSMCs) were pretreated with various concentrations of N-acetylcysteine for 2 h and then incubated with 100 μg·mL^{-1} of LPS for 6 h or 24 h. Cell lysates were analysed by Western blot using an anti-cPLA$_2$, anti-COX-2 or anti-β-actin Ab. (B) Cells were pretreated with 100 μM of N-acetylcysteine and then incubated with 100 μg·mL^{-1} LPS for 4 h. Expression of cPAL$_2$ or COX-2 mRNA was analysed by RT-PCR. (C, D) HTSMCs deprived of serum were pretreated with or without N-acetylcysteine (NAC; 100 μM) for 2 h and then incubated with 100 μg·mL^{-1} LPS for the indicated time intervals or 15 min. The amount of intracellular ROS was determined by using H_2DCFDA fluorescence dye. Data are expressed as mean ± SEM of five independent experiments ($n = 5$). *$P < 0.05$, #$P < 0.01$ as compared with the cells exposed to vehicle alone.

from LPS-challenged HTSMCs. We found that LPS decreased the amount of HuR in the nuclear fraction and increased that in the cytosolic fraction (Figure 5B). These data indicate that LPS stimulates the cytosolic accumulation of HuR in HTSMCs. To determine whether NADPH oxidase/ROS generation and MAPKs activation participate in this LPS-induced activation of HuR, HTSMCs were pretreated with an ROS scavenger (NAC), inhibitors of NADPH oxidase (DPI and APO), MEK1/2 (PD98059 and U0126), p38 MAPK (SB202190) or JNK1/2 (SP600125) for 1 h and then incubated with LPS (100 μg·mL^{-1}) for 90 min. The cytosolic fraction was prepared and analysed by Western blot using an anti-HuR antibody. We found that LPS-induced cytosolic accumulation of HuR was significantly inhibited by pretreatment with NAC, DPI, APO, PD98059, U0126, SB202190 or SP600125 (Figure 5C). To confirm these results, immunofluorescent staining also revealed that LPS-stimulated accumulation of HuR was significantly attenuated by pretreatment of HTSMCs with NAC, DPI, APO, PD98059, U0126, SB202190 or SP600125 (Figure 5D). Taken together, these data suggest that LPS-induced cPLA$_2$ and COX-2 expression is mediated through an NADPH oxidase/ROS/MAPKs-dependent activation of HuR.

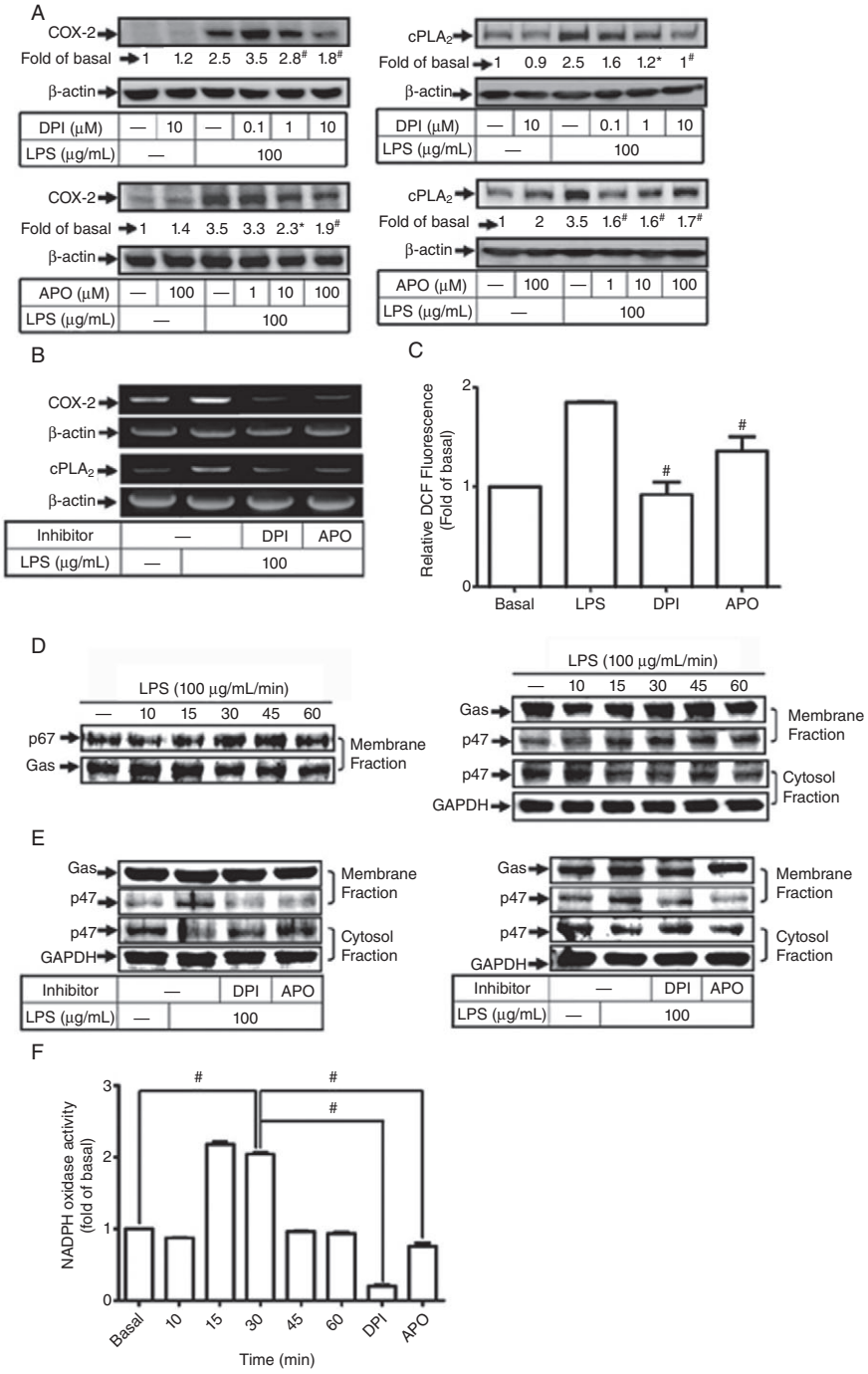

Figure 3

Involvement of NADPH oxidase in lipopolysaccharide (LPS)-stimulated cyclooxygenase-2 (COX-2) and cytosolic phospholipase A_2 ($cPLA_2$) expression. (A) Human tracheal smooth muscle cells (HTSMCs) were pretreated with various concentrations of diphenyleneiodonium chloride (DPI) or apocynin (APO) for 1 h and then incubated with 100 μg·mL^{-1} LPS for 6 h or 24 h. Cell lysates were analysed by Western blot using an anti-$cPLA_2$, anti-COX-2 or anti-β-actin Ab. (B) Cells were pretreated with DPI (10 μM) or APO (100 μM) for 1 h and then incubated with 100 μg·mL^{-1} LPS for another 4 h. mRNA expression of $cPLA_2$ and COX-2 was detected by RT-PCR. (C) HTSMCs were pretreated with DPI (10 μM) or APO (100 μM) for 1 h and then incubated with 100 μg·mL^{-1} LPS for 15 min. The amount of intracellular reactive oxygen species (ROS) was determined by using H_2DCFDA fluorescent dye. (D, E) HTSMCs were pretreated without or with DPI (10 μM) or APO (100 μM) for 1 h and then incubated with 100 μg·mL^{-1} LPS for the indicated time intervals or 15 min. Cytosolic and membrane fractions of cell lysates were prepared and analysed by Western blot using an anti-p47, anti-p67, anti-Gαs or anti-GAPDH Ab. Gαs and GAPDH were used as marker proteins for membrane and cytosolic fractions respectively. (F) HTSMCs were stimulated with 100 μg·mL^{-1} LPS for the indicated time intervals or pretreated with DPI (10 μM) or apocycin (100 μM) for 1 h and then incubated with 100 μg·mL^{-1} LPS for 30 min. The activity of NADPH oxidase was determined. Data are expressed as mean ± SEM of five independent experiments ($n = 5$). #$P < 0.01$ as compared with the cells exposed to LPS or vehicle alone, as indicated.

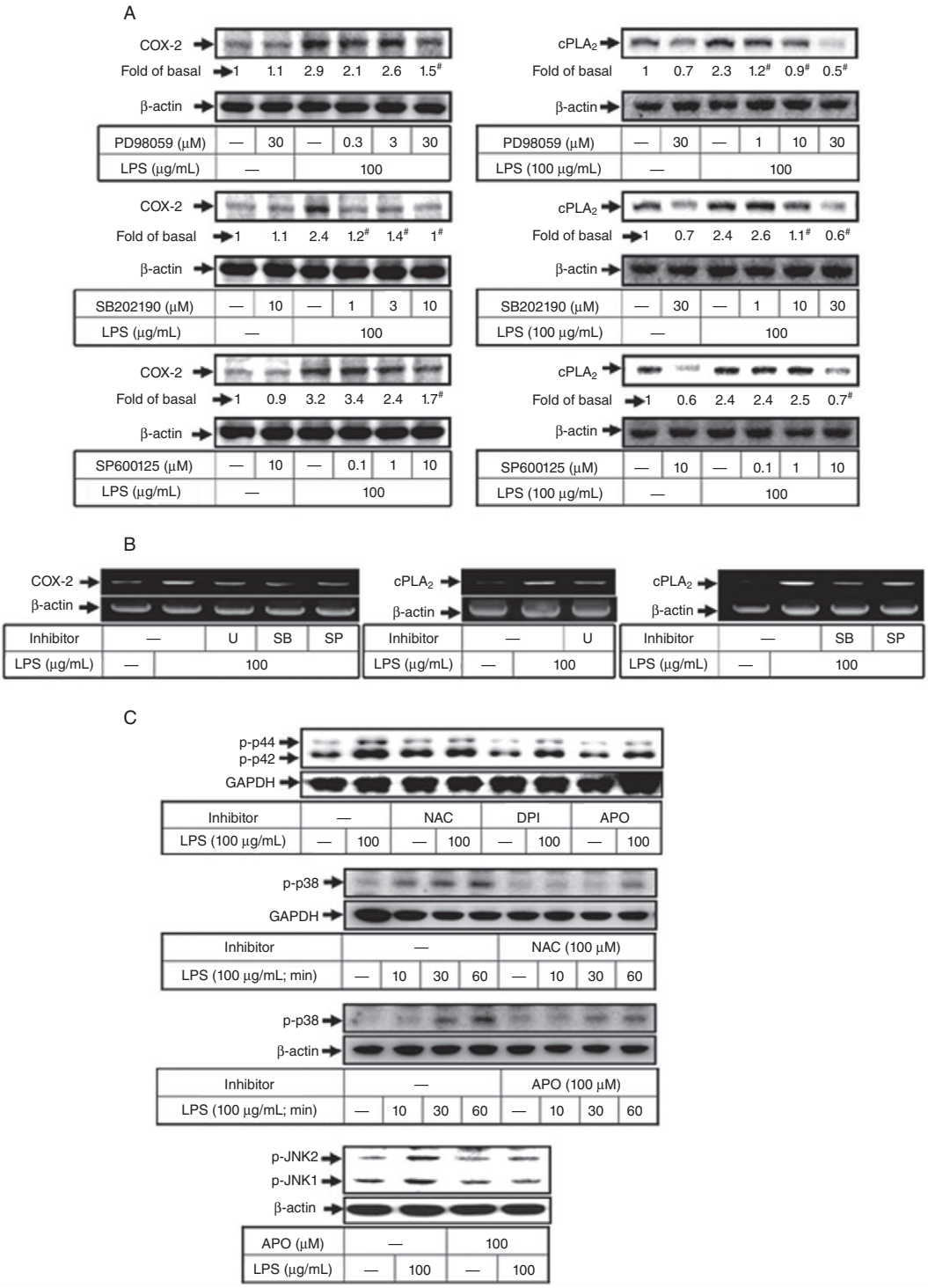

Figure 4

Lipopolysaccharide (LPS) induces cyclooxygenase-2 (COX-2) and cytosolic phospholipase A_2 (cPLA$_2$) expression through reactive oxygen species (ROS)-activated MAPKs in human tracheal smooth muscle cells (HTSMCs). (A) HTSMCs were pretreated with various concentrations of PD98059, SB203580 or SP600125 for 1 h and then incubated with 100 μg·mL^{-1} LPS for 6 h or 24 h. Cell lysates were analysed by Western blot using an anti-COX-2, anti-cPLA$_2$ or anti-β-actin Ab. (B) Cells were pretreated with 30 μM PD98059, 30 μM SB202190 or 10 μM SP600125 for 1 h and then incubated with 100 μg·mL^{-1} LPS for 4 h. mRNA expression of COX-2 and cPLA$_2$ was determined by RT-PCR. (C) HTSMCs were pretreated with N-acetylcysteine (100 μM), diphenyleneiodonium chloride (DPI) (10 μM) or apocynin (APO) (100 μM) for 1 h and then incubated with 100 μg·mL^{-1} LPS for 10 min or the indicated time intervals. MAPKs in cell lysates were determined by Western blot analysis using an anti-phospho-p42/p44 MAPK, anti-phospho-p38 MAPK, anti-phospho-JNK or anti-β-actin Ab. Similar results were obtained with five independent experiments (n = 5).

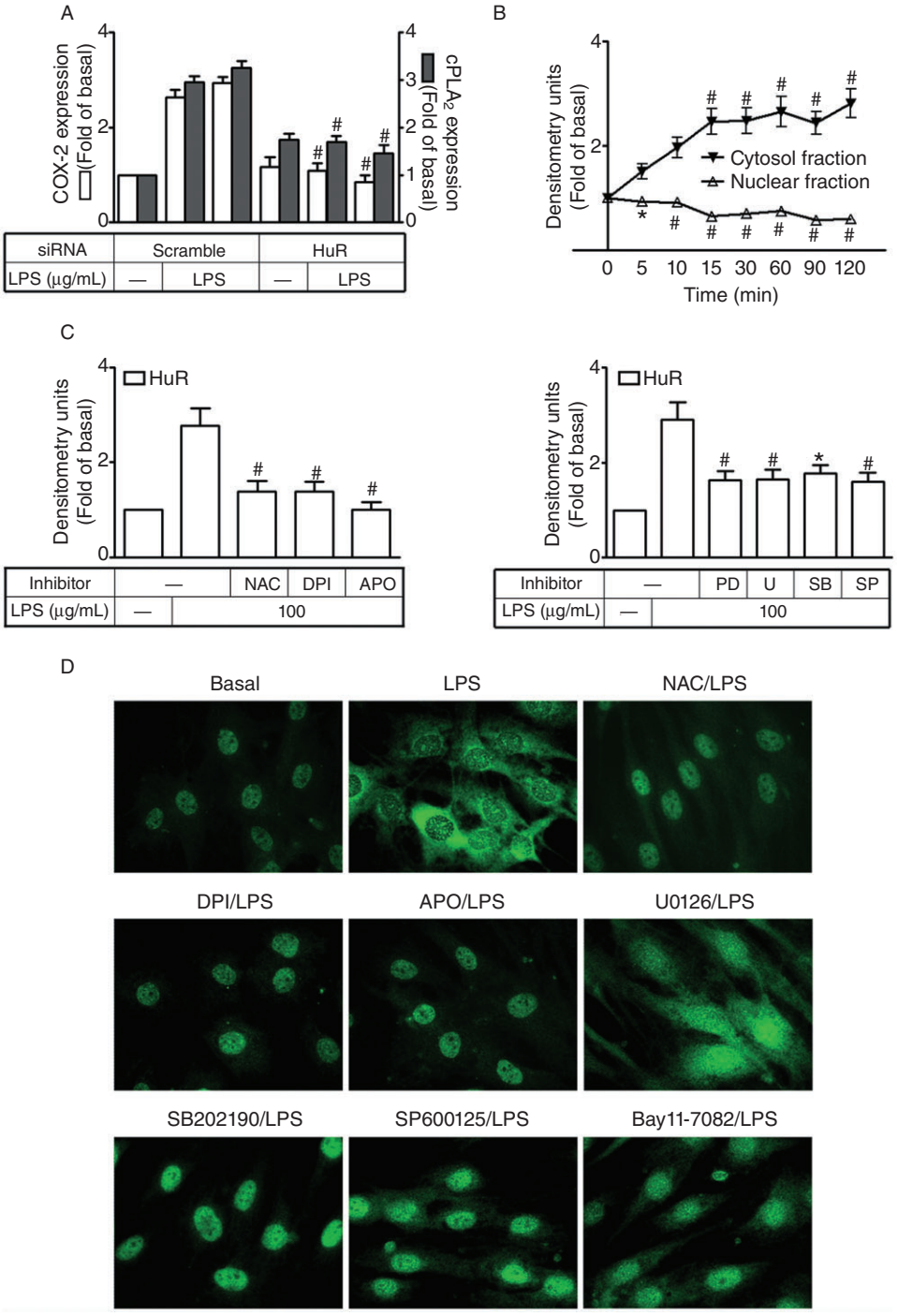

Figure 5

Involvement of HuR in lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) and cytosolic phospholipase A_2 ($cPLA_2$) expression in human tracheal smooth muscle cells (HTSMCs). (A) HTSMCs were transfected with HuR siRNA for 24 h, deprived of serum for 48 h, and then incubated with 100 μg·mL^{-1} LPS for 6 h or 24 h. Expression of $cPLA_2$, COX-2 and HuR was determined by Western blot analysis using an anti-COX-2, anti-$cPLA_2$, anti-HuR or anti-β-actin Ab. (B) HTSMCs deprived of serum were stimulated with 100 μg·mL^{-1} LPS for the indicated time intervals. Nuclear and cytosolic fractions were prepared and analysed by Western blot using an anti-HuR, anti-lamin A or anti-β-actin Ab. (C) Cells were pretreated with N-acetylcysteine (100 μM), diphenyleneiodonium chloride (DPI) (10 μM), apocynin (APO) (100 μM), PD98059 (30 μM), U0126 (10 μM), SB202190 (30 μM) or SP600125 (10 μM) for 1 h and then incubated with 100 μg·mL^{-1} LPS for another 90 min. Cell fraction assays were performed and cytosolic fractions of cells were analyzed by Western blot with anti-HuR and anti-β-actin actin. Data are expressed as mean ± SEM of five independent experiments (n = 5). *P < 0.05, #P < 0.01 as compared with the cells exposed to LPS. (D) HTSMCs were pretreated with N-acetylcysteine (NAC; 100 μM), DPI (10 μM), APO (100 μM), U0126 (10 μM), SB202190 (30 μM) or SP600125 (10 μM) for 1 h and then stimulated with 100 mg·mL^{-1} LPS for 60 min. Immunofluorescence staining was performed using an anti-HuR Ab. Similar results were obtained with five independent experiments (n = 5).

LPS stimulates the physical association between HuR and MAPKs

To further demonstrate that activation of p42/p44 MAPK, p38 MAPK or JNK1/2 is associated with the activation of HuR, an immunoprecipitation assay was performed using an anti-HuR, anti-p44, anti-p38 or anti-JNK2 Ab. Firstly, HTSMCs were incubated with 100 μg·mL^{-1} LPS for the indicated time intervals and the immunoprecipitation assay was performed using an anti-HuR Ab. We found that LPS stimulated a physical interaction between HuR and p42, p38 or JNK2 in a time-dependent manner with the maximal response occurring within 60 min (Figure 6A). To determine whether the interaction between HuR and MAPKs was attenuated by their respective inhibitors, cells were pretreated with inhibitors of MEK1/2 (U0126), p38 MAPK (SB202190) or JNK1/2 (SP600125) for 1 h and then incubated with 100 μg·mL^{-1} LPS for 60 min. As shown in Figure 6B–D, LPS-stimulated physical association between HuR and p42/p44 MAPK, p38 MAPK or JNK2 was reduced by U0126, SB202190 or SP600126. Moreover, pretreatment with an ROS scavenger (NAC), or NADPH oxidase inhibitors (DPI or APO), also significantly reduced the physical interaction between HuR and p42/p44 MAPK, p38 MAPK or JNK (Figure 6E). These data suggest that the LPS-induced physical association between HuR and MAPKs is mediated through an NADPH oxidase/ROS generation-dependent cascade.

The involvement of HuR in LPS-induced cPLA$_2$ and COX-2 expression

To further confirm the involvement of HuR in the expression of cPLA$_2$ and COX-2 induced by LPS, an HuR plasmid was constructed and transiently transfected into HTSMCs. Transfection with HuR plasmid led to an increased expression of HuR protein and subsequently enhanced LPS-induced expression of COX-2 and cPLA$_2$ protein, as revealed by Western blot analysis (Figure 7A). Similarly, the expression of COX-2 and cPLA$_2$ mRNA was also up-regulated in the HTSMCs transfected with HuR plasmid (Figure 7B). The involvement of HuR in the LPS-induced responses was further confirmed by transfection with HuR siRNA. As shown in Figure 7C and D, transfection with HuR siRNA significantly reduced LPS-induced COX-2 and cPLA$_2$ mRNA expression, as determined by RT-PCR and real-time PCR respectively. The stabilities of cPLA$_2$ and COX-2 mRNA were not significantly affected by addition of actinomycin D 4 h after treatment with LPS (Figure 7D). These data further suggest that HuR plays an important role in modulating LPS-stimulated COX-2 and cPLA$_2$ expression in HTSMCs.

Discussion and conclusions

Inflammation is an innate immunity of numerous airway diseases including asthma and COPD. COX-2 and cPLA$_2$ are key enzymes in mediating the conversion of lipids to PGs and LTs which further regulate the process of inflammation (Samad *et al.*, 2002). However, the action of LPS, a candidate of these bacteria-induced allergies, in regulating the expression of COX-2 and cPLA$_2$ in HTSMCs has not been completely elucidated. Here we showed that LPS stimulates the expression of COX-2 and cPLA$_2$ genes via the activation of NADPH oxidase, generation of ROS and phosphorylation of MAPKs. In addition, we showed that overexpression of HuR positively up-regulated COX-2 and cPLA$_2$ expression in LPS-challenged HTSMCs knocked-down HuR expression, induced by transfection with HuR siRNA significantly reduced COX-2 and cPLA$_2$ expression in these HTSMCs. Our results obtained by cell fraction assay, immunofluorescent staining and immunoprecipitation assays also revealed that p42/p44 MAPK, p38 MAPK and JNK participate in LPS-mediated nucleocytoplasmic shuttling of HuR. Western blot analysis revealed that activation of these MAPKs was regulated by activated NADPH oxidase-dependent generation of ROS. Thus, we conclude that in HTSMCs, LPS induces COX-2 and cPLA$_2$ expression by promoting the cytosolic export of HuR via NADPH oxidase/ROS-dependent phosphorylation of MAPKs.

HTSMCs have been demonstrated to play important roles in orchestrating and perpetuating airway inflammation, remodelling and fibrosis in chronic airway diseases (Tliba and Panettieri, 2008). Phenotype transition of HTSMCs from contractile units to synthetic function is a major signature of the pathology of airways during disease. Here we showed that LPS induces HTSMCs to express COX-2 and cPLA$_2$, which accompanied an increased secretion of PGE$_2$ (Figure 1). This accords with the higher PGE$_2$ levels found in the sputum of patients with eosinophilic bronchitis (Sastre *et al.*, 2008), and also with results obtained with RAW264.7 macrophages showing that LPS induces the production of inflammatory mediators such as PGE$_2$ and COX-2 (Ci *et al.*, 2009). However, the functions of PGs in regulating the outcome of inflammation are still controversial. Thus, understanding the mechanisms associated with the generation of PGs will help to improve the development of specific drug targets.

HuR, a ubiquitously expressed membrane of ELAV family, has been shown to participate in mediating the stabilization of a broad spectrum of mRNAs coding for cell-cycle regulators, cytokines, growth factors, tumour suppressors, protooncogenes, apoptosis regulatory proteins and various inflammatory enzymes (Doller *et al.*, 2008b). In this study, induction of COX-2 and cPLA$_2$ expression by LPS were found to be dependent on the mRNA-binding protein HuR, which accumulated in the cytoplasm upon exposure of HTSMCs to LPS (Figure 5). Both mRNA binding activity and nucleocytoplasmic shuttling of HuR are known to be regulated by several protein kinases such as MAPKs. In the present study, these MAPKs were demonstrated to enhance HuR-dependent mRNA stabilization in LPS-challenged HTSMCs (Figure 6). In fact, it was recently demonstrated that HuR is a direct substrate of p38 MAPK which stimulates phosphorylation of HuR and thus enhances its mRNA stabilizing activity (Lafarga *et al.*, 2009). Here we found that overexpression of HuR siRNA significantly attenuated LPS-induced COX-2 and cPLA$_2$ expression, while overexpression of HuR protein significantly promoted LPS-mediated COX-2 and cPAL$_2$ expression (Figures 5A and 7), suggesting that HuR contributes to the inflammatory effect of LPS in HTSMCs. Moreover, treatment with actinomycin D had no effect on LPS-mediated COX-2 and cPLA$_2$ mRNA expression, which was reduced in HTSMCs transfected with HuR siRNA (Figure 7C and D), indicating the involvement of HuR in these LPS-mediated responses.

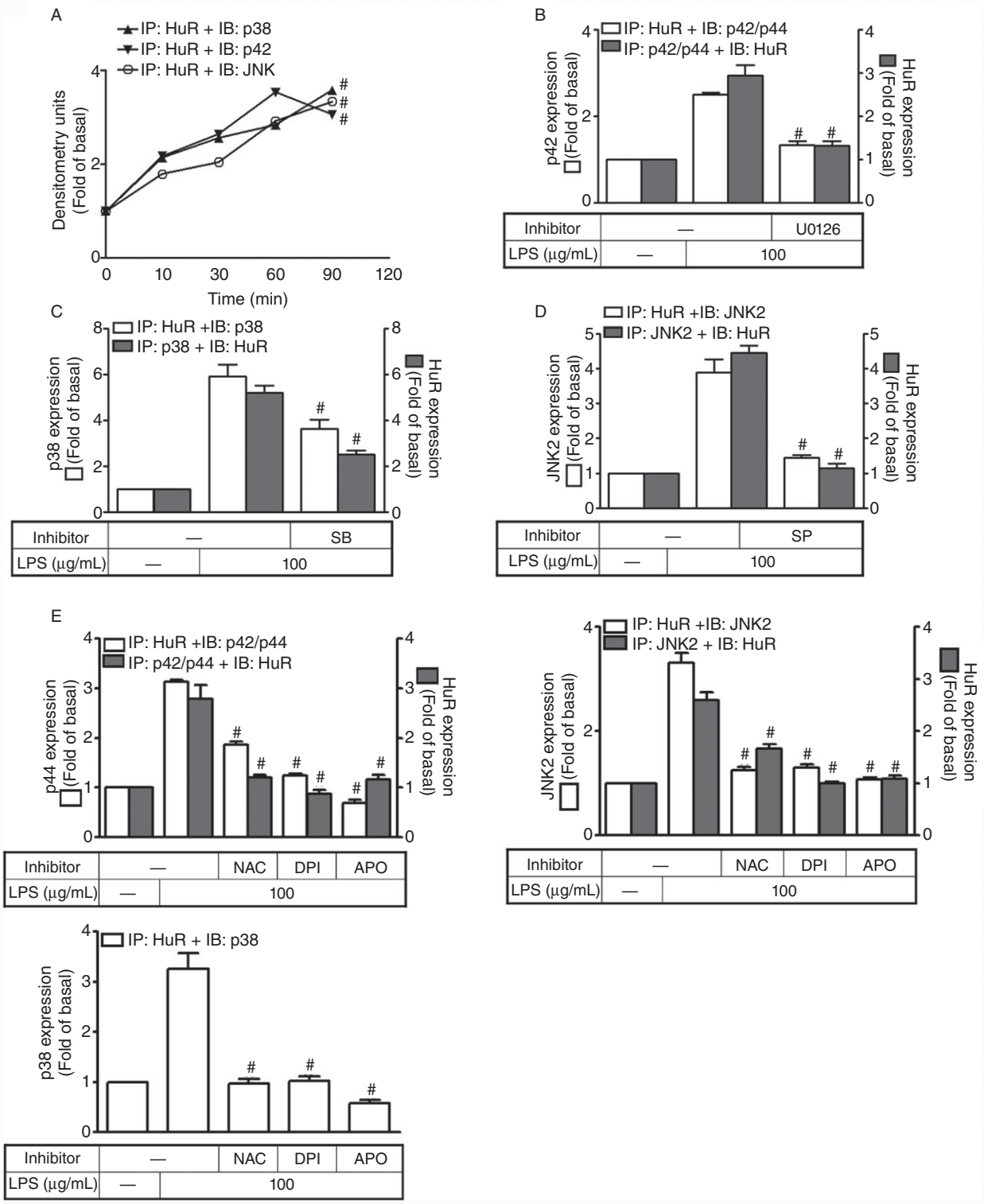

Figure 6

Lipopolysaccharide (LPS) stimulated the physical interaction between HuR and MAPKs in human tracheal smooth muscle cells (HTSMCs). (A–D) HTSMCs deprived of serum were incubated with 100 μg·mL^{-1} LPS for the indicated time intervals or pretreated with 10 μM U0126, 30 μM SB202190 or 10 μM of SP600125 for 1 h and then incubated with LPS for another 1 h. The cell lysates were subjected to immunoprecipitation with an anti-HuR, anti-p42/p44 MAPK, anti-p38 MAPK or anti-JNK2 Ab, and then the immunoprecipitates were analysed by Western blot using an anti-HuR, anti-p42 MAPK, anti-p38 MAPK or anti-JNK2. (E) HTSMCs were pretreated with N-acetylcysteine (NAC; 100 μM), diphenyleneiodonium chloride (DPI; 10 μM) or apocynin (APO; 100 μM) for 1 h and then stimulated by LPS (100 μg·mL^{-1}) for 60 min. The cell lysates were analysed as described in (A–D). Data are expressed as mean ± SEM of five independent experiments ($n = 5$). #$P < 0.01$ as compared with the cells exposed to LPS or vehicle alone.

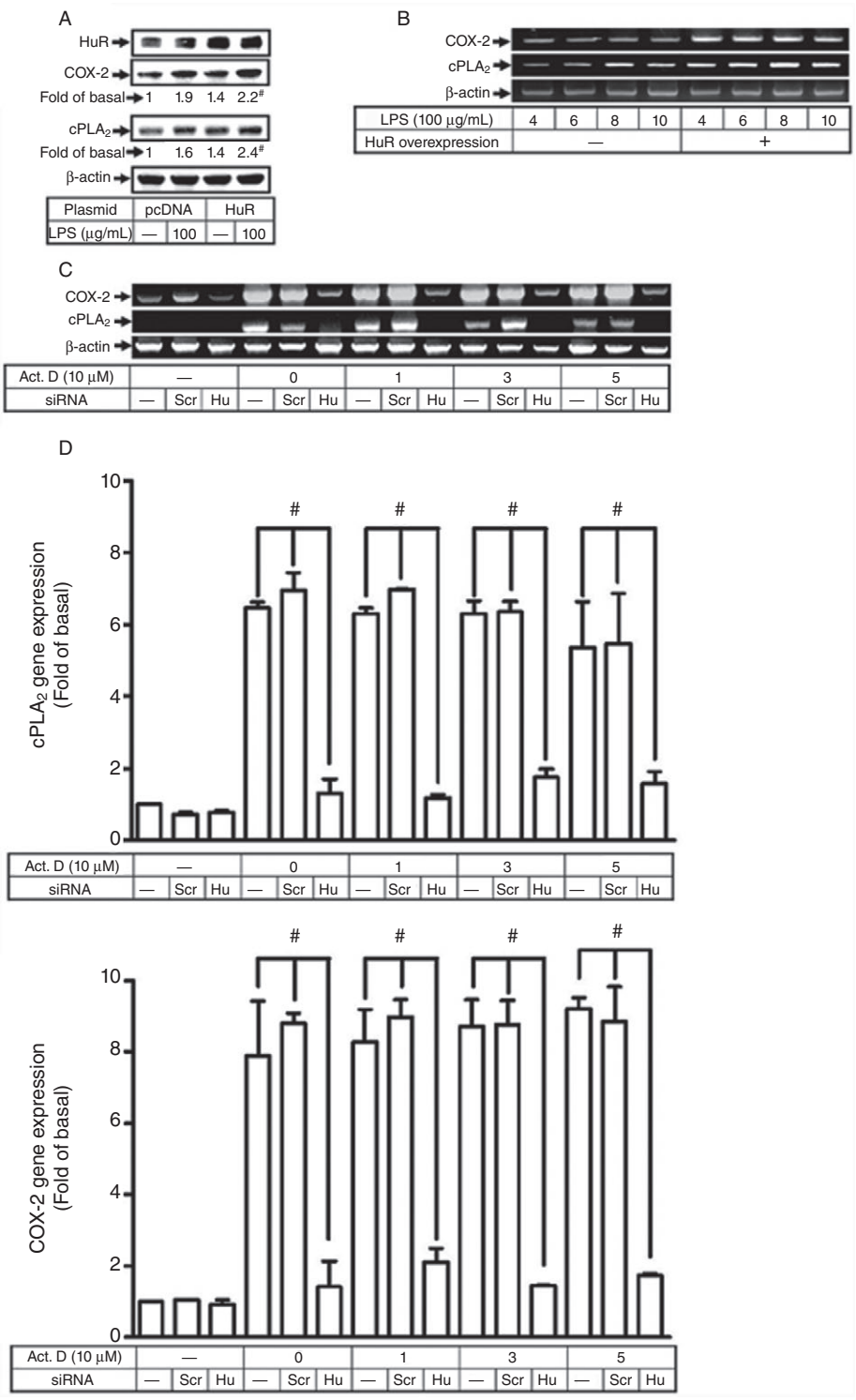

Figure 7

Involvement of HuR in lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) and cytosolic phospholipase A$_2$ (cPLA$_2$) expression. (A) Human tracheal smooth muscle cells (HTSMCs) were transfected with HuR plamid or pcDNA for 24 h, serum-starved for 48 h, and then incubated by 100 μg·mL^{-1} of LPS for 6 h or 24 h. Expression of cPLA$_2$, COX-2 and HuR was determined by Western blot using an anti-COX-2, anticPLA$_2$ or anti-HuR Ab. (B) HTSMCs were transfected with HuR plasmid and then stimulated with 100 μg·mL^{-1} of LPS for the indicated time periods. mRNA expression of COX-2 and cPLA$_2$ was determined by RT-PCR. (C, D) HTSMCs were transfected with HuR siRNA for 24 h, serum-starved for 48 h, incubated with LPS (100 μg·mL^{-1}) for 4 h, and then added with 1 μM of transcription inhibitor, actinomycin D (Act.D) for the indicated time intervals. mRNA was extracted, and the expression of COX-2 and cPLA$_2$ were analysed by RT-PCR and real-time PCR respectively. Data are expressed as mean ± SEM of five independent experiments (n = 5). #P < 0.01, as compared with the respective values of cells incubated with vehicle alone.

Similarly, HuR have been shown to be involved in the expression of plasminogen activator inhibitor-1 and COX-2 in response to angiotensin-II in kidneys (Doller *et al.*, 2009). In addition, ultraviolet B stimulates COX-2 expression by modifying the activity of HuR in a human keratinocyte cell line HaCaT (Fernau *et al.*, 2009).

Moreover, the localization of HuR in cytoplasm is important for the stabilization of target gene mRNA. However, the exact mechanisms that mediate the nucleocytoplasmic shuttling of HuR are still not known. Here we found that in HTSMCs, LPS induces the nuclear export of HuR via MAPKs (Figure 5B and D). In addition, we also showed that HuR may complex with p42/p44 MAPK, p38 MAPK or JNK1/2, which was attenuated by pretreatment with the inhibitors of MAPKs, as revealed by an immunoprecipitation assay (Figure 6). These results suggest that activated MAPKs interact with HuR and regulate the cytosolic localization of HuR, and are consistent with the findings of Lafarga *et al.* (2009) who by investigating the effects of γ-radiation on p21-related cell cycle arrest showed that activated p38 MAPK regulates the cytoplasmic accumulation of HuR. In addition, the increase in the accumulation of HuR in cytoplasma has also been found to be regulated by activated JNK and related to tamoxifen resistance in breast cancer cells (Hostetter *et al.*, 2008).

Recently, oxidative stress has been found to have a role in various inflammation-related pathologies including asthma. Here we found that pretreatment of HTSMCs with NADPH oxidase inhibitors or an ROS scavenger also attenuated the phosphorylation of MAPKs and inhibited the interaction between HuR and MAPKs (Figures 4C and 6E), indicating that NADPH oxidase and ROS contribute to the association between MAPKs and HuR and induce the nuclear export of HuR. In addition, LPS stimulation directly induced membrane localization of NADPH oxidase subunits, p47 and p67, and increased NADPH oxidase activity, which was associated with an increased intracellular ROS level (Figures 3D, F and 2C). LPS-mediated membrane localization of $p67^{phox}$ and $p47^{phox}$ was attenuated by pretreatment with either DPI or APO (Figure 3E), consistent with the results obtained by Barbieri *et al.* (2004) and Nishikawa *et al.* (2007). In addition, pretreatment with NADPH oxidase inhibitors or an ROS scavenger significantly attenuated the LPS-induced COX-2 and $cPLA_2$ expression (Figures 2A, B and 3A, B). These results indicate that NADPH oxidase-dependent generation of ROS may mediate COX-2 and $cPLA_2$ expression by activating MAPKs and promoting the cytoplasmic accumulation of HuR, consistent with results obtained in human keratinocyte HaCaT cells and hepatocellular carcinoma cells (Lim *et al.*, 2009; Liu *et al.* 2009). Results obtained with macrophage RAW264.7 cells have also indicated that LPS mediates the activation of the NADPH oxidase/superoxide production that results in innate immune responses (Check *et al.*, 2009).

In conclusion, our results indicate that LPS promotes COX-2 and $cPLA_2$ gene expression via an increase in the cytoplasmic accumulation of HuR, which is involved in mediating the stability of mRNA. The mechanisms underlying LPS-stimulated nucleocytoplasmic shuttling of HuR were mediated through the activation of NADPH oxidase, generation of ROS and phosphorylation of MAPKs. We showed that in HTSMCs LPS induces the membrane localization of p47 and p67 as well as the activation of NADPH oxidase. Moreover, the activation of NADPH oxidase increased intracellular ROS levels in LPS-challenged HTSMCs and the increased ROS led to p42/p44 MAPK, p38 MAPK and JNK phosphorylation and their physical association with HuR, which resulted in cytoplasmic accumulation of HuR. Thus, in HTSMCs, the expression of COX-2 and $cPLA_2$ may be regulated by NADPH oxidase/ROS/MAPKs-dependent cytoplasmic accumulation of HuR in response to LPS. Manipulation of HuR expression may provide a novel strategy to deal with inflammation-related pathologies.

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Conflicts of interest

The authors have no financial conflict of interest.

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