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LPS-induced Toll-like receptor 4 signalling triggers cross-talk of apoptosis signal-regulating kinase 1 (ASK1) and HIF-1α protein

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Abstract Toll-like receptor 4 (TLR4) is required for recognition of lipopolysaccharide (LPS) of Gram-negative bacteria and induction of the innate immune response to them. Nevertheless, the involvement of some crucial pathways in TLR4 signalling is poorly understood. Here, we report that LPS-induced TLR4 signalling triggers cross talk of HIF-1α and ASK1 in THP-1 human myeloid monocytic leukaemia cells. Both pathways are activated via redox-dependent mechanism associated with tyrosine kinase/phospholipase C-1γ-mediated activation of protein kinase C α/β, which are known to activate NADPH oxidase and the production of reactive oxygen species that activate both HIF-1α and ASK1. ASK1 contributes to the stabilisation of HIF-1α, most likely via activation of p38 MAP kinase.
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Keywords: Toll-like receptor; Inflammation and innate immunity; HIF-1α; Apoptosis; MAP kinase cascade

1. Introduction

Human inflammatory/innate immune reactions are initiated via recognition of pathogen-associated molecular patterns by specific receptors. Toll-like receptors (TLRs) are the key pattern recognition receptors that allow cells to specifically detect pathogens by recognising structural components shared by many bacteria, viruses and fungi [1]. TLRs lie at the core of resistance to disease, initiating most of the phenomena that occur in the course of infection [2–4]. TLR4 is one of the most physiologically important TLRs initially identified as the lipopolysaccharide (LPS) recognising receptor required for detection of Gram-negative bacteria, which have LPS as an integral part of outer cell membrane. TLR4 is expressed in different cell types including myeloid cells, which are the key effectors of innate immune responses [5]. It has recently been reported that hypoxia-inducible factor 1α (HIF-1α) an inducible subunit of heterodimeric HIF-1 transcription complex consisting of two subunits (HIF-1α and HIF-1β) [6] is accumulated in response to LPS [7,8]. HIF-1α is rapidly accumulated in cells exposed to hypoxia [9] or some non-hypoxic stimuli like inflammatory mediators, cytokines, reactive oxygen (ROS) and nitrogen (RNS) species (reviewed in [9,10]). Other vice HIF-1α protein undergoes rapid ubiquitination followed by proteasomal degradation [9]. HIF-1α plays pivotal role in mediating angiogenesis, glycolysis and cell adhesion [9], which are critical for inflammation. However, it is still unclear whether LPS-dependent HIF-1α accumulating effect is achieved via TLR4. Basic biochemical mechanisms involved in LPS-dependent HIF-1α accumulation are not clear as well. One of possible mechanisms is the cross-talk of HIF-1α and apoptosis signal-regulating kinase 1 (ASK1) associated with generation of ROS/RNS. The most recent data suggest a crucial role of ASK1 (active form of the kinase interacts with tumour necrosis factor receptor associated factor 6 (TRAF6) forming catalytically active complex) and its downstream pathway in transduction of signals from TLR4 [11,12] including secretion of pro-inflammatory cytokines. ASK1 is selectively required for TLR4-dependent activation of p38 MAP kinase [11]. In normal cells ASK1 is directly inhibited by thioredoxin (Trx), a 12-kDa protein ubiquitously expressed in all living cells. Upon modification (for example oxidation) of Trx reactive thiol groups ASK1 dissociates and undergoes activation [14]. Despite it is known that ROS are involved in TLR4-mediated activation of ASK1 [11], there are no data concerning basic molecular mechanisms of ASK1 activation by active TLR4. Furthermore, p38 activated by ASK1 is known to phosphorylate and therefore support ischemic accumulation of HIF-1α protein [15]. There is, however, no evidence concerning involvement of ASK1-p38 cascade in LPS-dependent accumulation of HIF-1α protein. In addition, TLR4 signalling is known to stimulate enzymatic production of RNS, which are known to activate both ASK1 and HIF-1α [13]. However, there is no evidence concerning participation of RNS in LPS-dependent activation of ASK1 and HIF-1α.

Here we report that LPS induces both HIF-1α accumulation/DNA-binding activity and activity of ASK1 in THP-1 human myeloid monocytic leukaemia cells in dose- and time-dependent manner. Pre-treatment of THP-1 cells with anti-TLR4 antibody followed by stimulation with LPS attenuated both HIF-1α accumulation and activation of ASK1. Pre-treatment of cells with tyrosine kinase inhibitor genistein, PI-specific phospholipase C inhibitor U73122, PKCα/β inhibitor Gö6983 and NADPH oxidase inhibitor diphenyleniodonium chloride (DPI) attenuated LPS-induced NADPH oxidase (Nox)-dependent ROS production and both HIF-1α accumulation/ASK1 activation when stimulated during with LPS. The same effect was observed when the cells were exposed to antioxidant *N*-acetylcysteine (NAC). Nitric oxide synthase inhibitor *N*-monomethyl arginine (NMMA) blocked LPS-induced RNS production but did not impact HIF-1α accumulation/ASK1 activity when the cells were exposed during 4 h to LPS. Transfection of THP-1 cells with dominant-negative isoform of

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ASK1 attenuated LPS-induced HIF-1α accumulation. The same effect was observed when the cells were pre-treated with p38 MAP kinase inhibitor SB203580 before 4 h of exposure to LPS. This effect was probably due to the inhibition of p38-dependent HIF-1α phosphorylation, which we have observed as well. We therefore suggest that TLR4 signalling leads to accumulation of HIF-1α protein via Nox-generated ROS followed by phosphorylation by ASK1-activated p38.

2. Materials and methods

2.1. Materials

Medium, fetal calf serum and supplements, caspase 3 colorimetric assay kit, DOTAP transfection reagent, GenElute™ palsmid purification kit, enhanced avian HS RT-PCR kit, GenElute™ mammalian total RNA miniprep kit, nitric oxide synthase inhibitor *N*-monomethyl arginine (NMMA), genistein (tyrosine kinase inhibitor), PI-specific PLC inhibitor U73122, PKCα/β inhibitor Gö6983, NADPH oxidase inhibitor diphenyleniodonium chloride (DPI) were purchased from Sigma (Suffolk, UK). p38 MAP kinase specific inhibitor SB203580 was bought from Alexis (Nottingham, UK). Maxisorp™ microtitre plates were obtained from Nunc (Roskilde, Denmark). Mouse monoclonal antibody to HIF-1α, mouse monoclonal antibody to TLR4, rabbit monoclonal antibody to ASK1, mouse monoclonal antibody to β-actin as well as rabbit polyclonal HRP-labeled antibody to mouse IgG and goat polyclonal HRP-labeled antibody to rabbit IgG were from Abcam (Cambridge, UK). All other chemicals were of the highest grade of purity and commercially available.

2.2. Cell culture and preparation of the whole cell extracts

THP-1 human leukemia monocytic macrophages were purchased from European collection of Cell Cultures (Salisbury, UK). Cells were grown in RPMI 1640 media supplemented with 10% fetal calf serum, penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml).

2.3. Plasmids

Plasmid encoding constitutively active human TLR4 (murine CD4 fused to human TLR4) [16] was generously provided by Professor Medzhitov (Yale University, New Haven, USA). Plasmid encoding hemagglutinin (HA)-tagged wild-type human ASK1 and ASK1 with kinase-dead domain (dominant-negative form), ASK1-KM was a kind gift of Professor Ichijo (University of Tokyo, Tokyo, Japan). Plasmids were amplified in *Escherichia coli* XL10 Gold® (Stratagene Europe, Amsterdam, The Netherlands) and purified using GenElute™ plasmid purification kit according to the manufacturers protocol. Purified plasmids were transfected into THP-1 cells using DOTAP transfection reagent according to the manufacturer's protocol.

2.4. Detection of HIF-1α protein

HIF-1α was determined by Western blot analysis, as previously described [17]. Briefly, cells were incubated for the times indicated, washed two times with ice-cold phosphate-buffered saline (PBS) and lysed in 200 μl of lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0). After centrifugation ($17000 \times g$, 15 min) the protein content in the supernatants was analysed (the same procedures applied to Western blot analysis of other proteins). Finally, 100 μg of protein was added to the same volume of 2× sample buffer (125 mM Tris–HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerine, 1 mM dithiothreitol (DTT), 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5% SDS–polyacrylamide gels and blotted to nitrocellulose membrane. Molecular weights were calibrated in proportion to the running distance of rainbow markers. Transblots were washed twice with TBS (50 mM Tris–HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween 20 before blocking unspecific binding with TBS plus 5% skim milk for 1 h. The HIF-1α (1:1000 in TBS plus 5% milk) antibody was added and incubated for 60 min at room temperature. Afterwards, nitrocellulose membranes were washed five times for 15 min with TBS containing 0.1% Tween 20. For protein detection, blots were incubated with goat secondary antibodies conjugated with horse radish peroxidase (1:1000 in TBS plus 5% milk) for 60 min, followed by ECL detection. Blots were stripped and re-probed with a rabbit monoclonal antibody against β-actin to confirm equal protein loading. Briefly, membranes were washed five times with TBS (50 mM Tris–HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween 20 then incubated for 1 h at room temperature with monoclonal antibody directed against β-actin followed by ECL detection.

2.5. Determination of HIF-1 DNA-binding activity

HIF-1 DNA-binding activity was measured by the method similar to the one described recently with our modifications [8]. 96-well maxisorp™ microtitre plate was coated with streptavidin and blocked with BSA as described before [18]. 2 pmol/well-biotinylated 2HRE-containing oligonucleotide were immobilised by 1 h incubation at room temperature. Plate was then washed five times with TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) followed by 1 h incubation with 20 μl/well of cell lysate at room temperature. Plate was again washed five times with TBST buffer and mouse anti-HIF-1α antibody (1:1000 in TBS plus 2% BSA) was added. After 1 h of incubation at room temperature plate was washed five times with TBST buffer and incubated with 1:1000 HRP-labelled rabbit anti-mouse IgG in TBST buffer and after extensive washing with TBST, the bound secondary antibody was detected by the peroxidase reaction (*ortho*-phenylenediamine/H_2O_2, Kem-En-Tek Diagnostics, Copenhagen, Denmark). The reactions were quenched after 10 min with an equal volume of 1 M H_2SO_4 and the colour development was measured in a microplate reader as the absorbance at 492 nm. DNA-binding activity of HIF-1 was calculated as a percentage of the value obtained when analysing lysates of non-treated cells (control).

2.6. Detection of ASK1 protein

ASK1 was measured by Western blot [19,20]. Cell lysate protein (100 μg) were added to the same volume of 2× sample buffer (125 mM Tris–HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5% SDS–polyacrylamide gels and blotted to nitrocellulose membrane. Molecular weights were calibrated in proportion to the running distance of rainbow markers. Transblots were washed twice with TBS (50 mM Tris–HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween 20 before blocking unspecific binding with TBS plus 5% skim milk for 1 h. The ASK1 (1:1000 in TBS plus 5% milk) monoclonal antibody was added and incubated for 60 min at room temperature. Afterwards, nitrocellulose membranes were washed five times for 15 min with TBS containing 0.1% Tween 20. For protein detection, blots were incubated with goat secondary antibodies conjugated with horse radish peroxidase (1:1000 in TBS plus 5% milk) for 60 min, followed by ECL detection.

2.7. Measurement of ASK1 kinase activity

The kinase activity of ASK1 was assayed by the method based on its immunoprecipitation followed by analysis of phosphorylation of exogenous substrate – myeline basic protein (MBP) and estimated in nmol of the phosphate transferred per 1 min onto the MBP per 1 mg protein as described previously [14,19–21].

2.8. Analysis of p38-dependent HIF-1α phosphorylation

p38-dependent HIF-1α phosphorylation was monitored using the following approach. 96-well maxisorp™ microtitre plate was coated with 2 μg/well anti-HIF-1α antibody and blocked with BSA as described before [18]. Plate was then washed five times with TBST buffer followed by 1 h incubation with 20 μl/well (room temperature) of lysates of THP-1 cells treated with for 4 h with LPS (to remove HIF-1α protein from the lysate) or 4 h with 100 μM cobalt chloride (well-known stimulator of HIF-1α accumulation [7]) after 30 min pre-treatment with p38 MAP kinase inhibitor 10 μM SB203580 (these lysates contain HIF-1α protein which is not phosphorylated by p38). Content of HIF-1α protein in each lysate was detected by Western blot as described above. After extensive washing with TBST buffer cell lysate lacking HIF-1α and containing active p38 (withdrawn from the wells containing lysates of LPS-treated cells) was transferred into

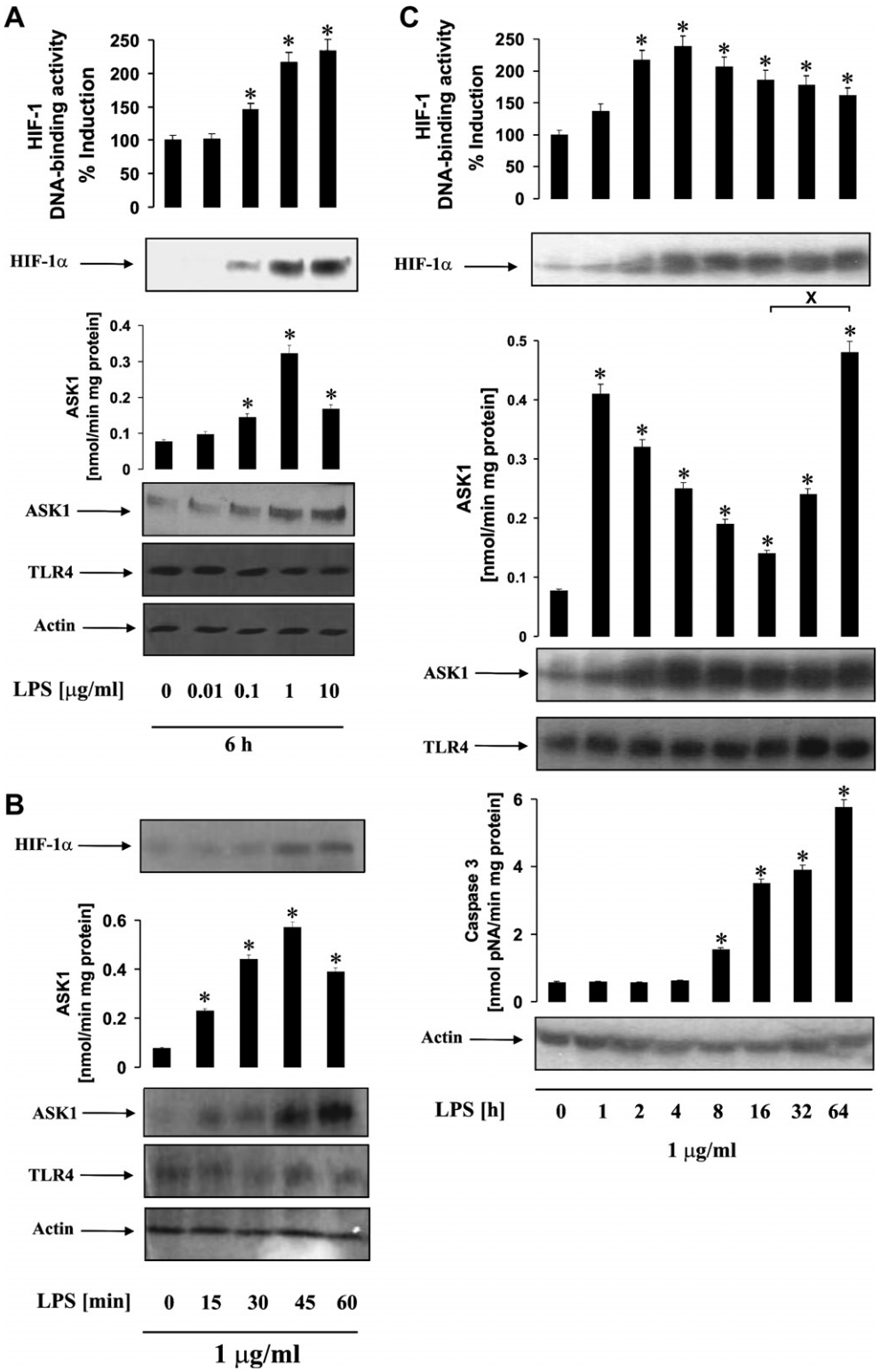

Fig. 1. LPS induces accumulation of HIF-1α protein and ASK1 activation. (A) THP-1 cells were stimulated during 6 h with 0.01, 0.1, 1 and 10 μg/ml LPS and accumulation (Western blot)/DNA-binding activity (ELISA) of HIF-1α protein as well as ASK1 protein stability (Western blot)/kinase activity (immunoprecipitation associated with MBP phosphorylation assay) were analysed as outlined in Section 2 (B) THP-1 cells were treated with 1 μg/ml LPS during 15, 30, 45 and 60 min and accumulation of HIF-1α protein, ASK1 protein stability/kinase activity were detected. (C) THP-1 cells were treated with 1 μg/ml LPS during 1, 2, 4, 8, 16, 32 and 64 h and accumulation/DNA-binding activity of HIF-1α protein, ASK1 protein stability/kinase activity and caspase 3 activity (colorimetric assay) were detected as described in Materials and methods. TLR4 level was also monitored (Western blot) and actin staining was used as a protein loading control in all experiments. Digital data are mean values ± S.D. of at least four individual experiments. $^{*}P < 0.01$ vs. control ($^{*}P < 0.01$ between two specific experiments). All Western-blot data are from one experiment representative of three that gave similar results.

the wells treated with lysates of SB203580/cobalt chloride treated cells. To some of the wells we added 10 μM SB203580 to block p38 activity. The incubation was performed during 1 h at room temperature and after extensive washing with TBST the amount of phosphate groups in isolated HIF-1α protein was quantified [22].

To confirm the role of p38 in HIF-1α phosphorylation we have stimulated THP-1 cells with 1 μg/ml LPS for 4 h and isolated p38 MAP kinase from them. For this purpose 96-well maxisorp™ microtitre plate was coated with 2 μg/well anti-p38 capture antibody and blocked with BSA as described before [18]. Plate was then washed five times with TBST buffer followed by 1 h incubation with 100 μl/well (room temperature) of lysates of THP-1 cells treated with for 4 h with 1 μg/ml LPS. To prepare isolated active HIF-1α protein we have treated THP-1 cells with 100 μM cobalt chloride in combination with 10 μM SB203580 to block p38 activity (cobalt chloride-dependent accumulation of HIF-1α protein does not require p38-dependent phosphorylation; as a control we used the cells which were not stimulated with cobalt chloride). 96-well maxisorp™ microtitre plate was coated with streptavidin and blocked with BSA as described before [18]. 2 pmol/well-biotinylated 2HRE-containing oligonucleotide were immobilised by 1 h incubation at room temperature. Plate was then washed five times with TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) followed by 1 h incubation with 100 μl/well of cell lysate at room temperature. Immobilised HIF-1α protein was eluted by glycine buffer (pH 2.5) and the pH was adjusted back to 7.3 by TBST buffer. Eluted HIF-1α protein was transferred to the wells containing immobilised p38 and incubated for 1 h with 10 μM ATP followed by detection of phosphate groups in it [17,19–21].

2.9. Detection of TLR4

TLR4 was assayed by Western blot analysis. Briefly, 100 μg of cell lysate protein were added to the same volume of 2× sample buffer (125 mM Tris–HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5% SDS–polyacrylamide gels and blotted to nitrocellulose membrane. Molecular weights were calibrated in proportion to the running distance of rainbow markers. Transblots were washed twice with TBS (50 mM Tris–HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween 20 before blocking unspecific binding with TBS plus 5% skim milk for 1 h. The TLR4 (1:1000 in TBS plus 5% milk) monoclonal antibody was added and incubated for 60 min at room temperature. Afterwards, nitrocellulose membranes were washed five times for 15 min with TBS containing 0.1% Tween 20. For protein detection, blots were incubated with goat secondary antibodies conjugated with horse radish peroxidase (1:1000 in TBS plus 5% milk) for 60 min, followed by ECL detection.

2.10. Measurement of caspase 3 activity

The activity of caspase 3 was assayed by colorimetric method based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) according to the manufacture's protocol.

2.11. Detection of Nox-dependent ROS generation and RNS production

Nox-dependent ROS generation was analysed as described before [8]. RNS production was analysed by Griess assay [23].

2.12. Measurement of HIF-1α mRNA by RT-PCR

Total RNA was isolated using GenElute™ mammalian total RNA miniprep kit, followed by HIF-1α mRNA reverse transcriptase-polymerase chain reaction (RT-PCR). Primer selection was as follows: HIF-1α, 5′-CTCAAAGTCGGACAGCCTCA-3′, 5′-CCCTGCAGTAGGTTTCTGCT-3′; actin, 5′-TGACGGGGTCACCCACA-CTGTGCCCATCTA-3′, 5′-CTAGAAGCATT-TGCGGTCGACGATGGAGGG-3′. Amplification program was as follows: 95 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min; 20 cycles; 72 °C, 10 min. Products were separated on 2% agarose gels and visualized with ethidium bromide.

2.13. Statistical analysis

Each experiment was performed at least three times and statistical analysis was done using the two-tailed Student's *t*-test. The statistical probability (*P*) expressed as $^{*}P < 0.01$. The normal distribution of data was checked.

3. Results and discussion

3.1. LPS induces accumulation of HIF-1α protein and ASK1 activation

We have first of all analysed dose-dependent LPS-mediated accumulation and DNA-binding activity of HIF-1α protein. It was found that stimulation of THP-1 cells with 0.1, 1 and 10 μg/ml LPS-induced accumulation of HIF-1α protein in concentration-dependent manner. As a consequence of the increase in HIF-1α protein levels, the increase (max ~2.5-fold) in HIF-1 DNA-binding activity was observed. ASK1 was activated by the same concentrations of LPS, however, the highest kinase activity was observed when the cells were exposed to 1 μg/ml LPS. TLR4 amount remained similar in all cases (Fig. 1A).

Further analysis was performed using 1 μg/ml concentration of LPS. We have found that HIF-1α protein starts accumulating and displays DNA-binding activity after 45 min of stimulation with LPS. Both accumulation/DNA-binding activity of HIF-1α protein are peaking after 4 h of stimulation with LPS. Then the amount of protein and its DNA-binding activity go down. However, HIF-1α protein remains stable and active upon even 64 h of stimulation with LPS (Fig. 1B and C).

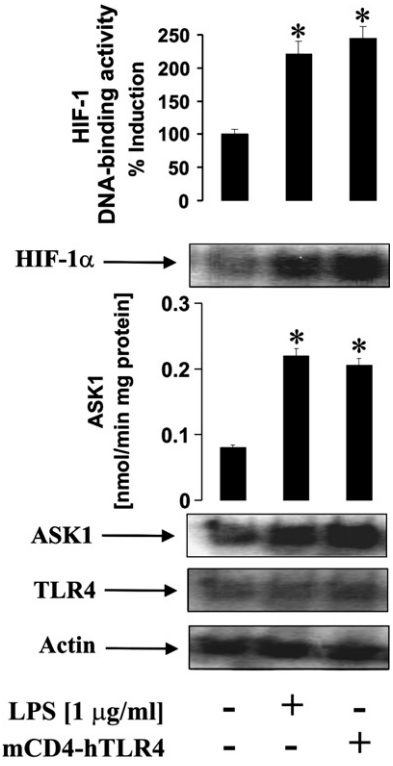

Fig. 2. TLR4 signalling leads to the accumulation of HIF-1α and activation of ASK1. We have transiently transfected THP-1 cells with constitutively active TLR4 (murine CD4 fused to human TLR4) and analysed accumulation (Western blot)/DNA-binding activity (ELISA) of HIF-1α protein as well as ASK1 protein stability (Western blot)/kinase activity (immunoprecipitation associated with MBP phosphorylation assay) as outlined in Section 2. TLR4 level was also monitored (Western blot). Actin staining was used as a protein loading control. LPS-induced effects were analysed as a positive control. Digital data are mean values ± S.D. of at least four individual experiments. $^{*}P < 0.01$ vs. control. All Western-blot data are from one experiment representative of three that gave similar results.

ASK1 gets stabilised in response to stimulation with LPS but the highest kinase activity was observed when the cells were stimulated during 45 min. Then ASK1 kinase activity went down but was again increased upon exposure of the cells to LPS during 32 and 64 h. This correlated with increase in caspase 3 activity suggesting induction of apoptotic cell death. No changes in the amount of TLR4 were observed in all cases (Fig. 1B and C).

3.2. TLR 4 signalling leads to the accumulation of HIF-1α and activation of ASK1

Most likely that the observed HIF-1α accumulating/ASK1 activating effects of LPS are achieved via activation of TLR4 and are part of TLR4 signalling. To confirm this we have transiently transfected THP-1 cells with constitutively active TLR4 (murine CD4 fused to human TLR4) [16] and analysed stabilisation/DNA-binding activity of HIF-1α and kinase activity/accumulation of ASK1. The results were similar to those observed upon stimulation of cells with LPS – HIF-1α accumulation/DNA-binding activity as well as ASK1 stability and kinase activity were increased (Fig. 2).

3.3. NADPH oxidase derived ROS and ASK1 but not RNS are critical for LPS-dependent TLR4-mediated HIF-1α accumulation

To further verify that LPS-dependent HIF-1α/ASK1 activation are TLR4-dependent we pre-treated the cells with 2 μg/ml TLR4 neutralising antibody during 2 h followed by 4 h of treatment with 1 μg/ml LPS [24]. In both cases both accumulation of HIF-1α and activation of ASK1 were attenuated suggesting that LPS-induced effects are TLR4-dependent (Fig. 3). One hour pre-treatment of cells with 100 μM NMMA followed by 4 h of stimulation with 1 μg/ml LPS blocked RNS production (from 180 ± 12% control to 89 ± 7% control as measured by Griess assay; the amount of RNS produced by non-stimulated THP-1 cells was considered as 100%) but did not impact HIF-1α accumulation or ASK1 activation. We hypothesised that NADPH oxidase (Nox) derived ROS are responsible to stabilise both HIF-1α and ASK1. During TLR4 signalling Nox is activated in the following way. Src tyrosine kinase activates of Bruton's tyrosine kinase (Btk) as well as other tyrosine kinases that interact with TIR-domain of TLR4 and stimulates recruitment of MyD88 and TIRAP

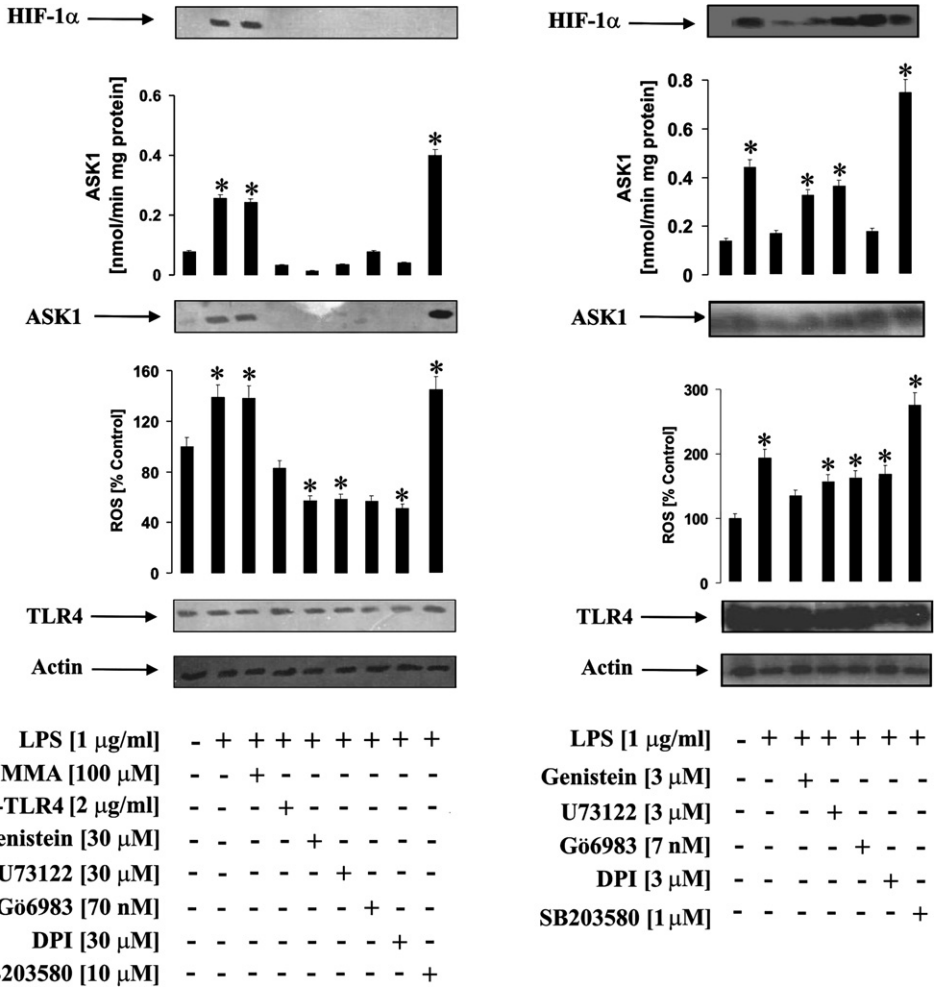

Fig. 3. NADPH oxidase derived ROS and ASK1 but not RNS are critical for LPS-dependent TLR4-mediated HIF-1α accumulation. THP-1 cells were pre-stimulated with respective inhibitors during 1 h (concentrations are outlined in the figure) and then stimulated during 4 h with 1 μg/ml LPS. Accumulation of HIF-1α protein (Western blot), ASK1 protein stability (Western blot)/kinase activity (immunoprecipitation associated with MBP phosphorylation assay), Nox-dependent and ROS production (luminometric assay) were analysed. TLR4 level was also monitored and actin staining was used as a protein loading control in all experiments. Digital data are mean values ± S.D. of at least four individual experiments. $^{*}P < 0.01$ vs. control. All Western-blot data are from one experiment representative of three that gave similar results.

(Mal) adaptor proteins required for TLR4 downstream signalling [25,26]. In addition, Btk and other tyrosine kinases activate PI-specific PLC-1γ known to release diacylglycerols (DAG) and PI that increases intracellular Ca^{2+} concentration [25–27]. Both DAG and Ca^{2+} activate PKCα/β known to phosphorylate $p47_{phox}$ subunit of Nox [27,28] leading to the enzyme activation followed by ROS production. To investigate involvement of this pathway in HIF-1α accumulation and ASK1 activation we have pre-treated the cells with 30 μM genistein (tyrosine kinase inhibitor), 30 μM U73122 (PI-specific PLC-1γ inhibitor), 70 nM Gö6983 (PKCα/β inhibitor) or 30 μM DPI (Nox inhibitor) followed by 4 h of stimulation with 1 μg/ml LPS. All listed inhibitors nullified both HIF-1α accumulation and ASK1 stabilisation/activation (Fig. 3). Results obtained confirm participation of Nox and Nox-activating pathway in both HIF-1α accumulation and activation of ASK1. No one of the inhibitors used changed the level of TLR4 produced in THP-1 cells (Fig. 3).

One hour pre-treatment of THP-1 cells with p38 MAP kinase inhibitor SB203580 completely attenuated LPS-dependent HIF-1α stabilisation when exposed 4 h to 1 μg/ml LPS (Fig. 3). Tenfold reduction of the concentrations of genistein, U73122, Gö6983, DPI and SB203580 decreased the inhibition of LPS-induced HIF-1α accumulating/ASK1 activating effect (Fig. 3). This treatment did not impact TLR4 level in THP-1 cells. Recently, it was found that p38-dependent HIF-1α phosphorylation is essential for protein stabilisation [15]. However, longer exposure to LPS by some reason reduces the critical role of p38 in HIF-1α accumulation. One could speculate that due to the increase HIF-1α in protein expression its post-translational stabilisation does not need to be enhanced by p38. To investigate critical role of ASK1 in LPS-dependent HIF-1α

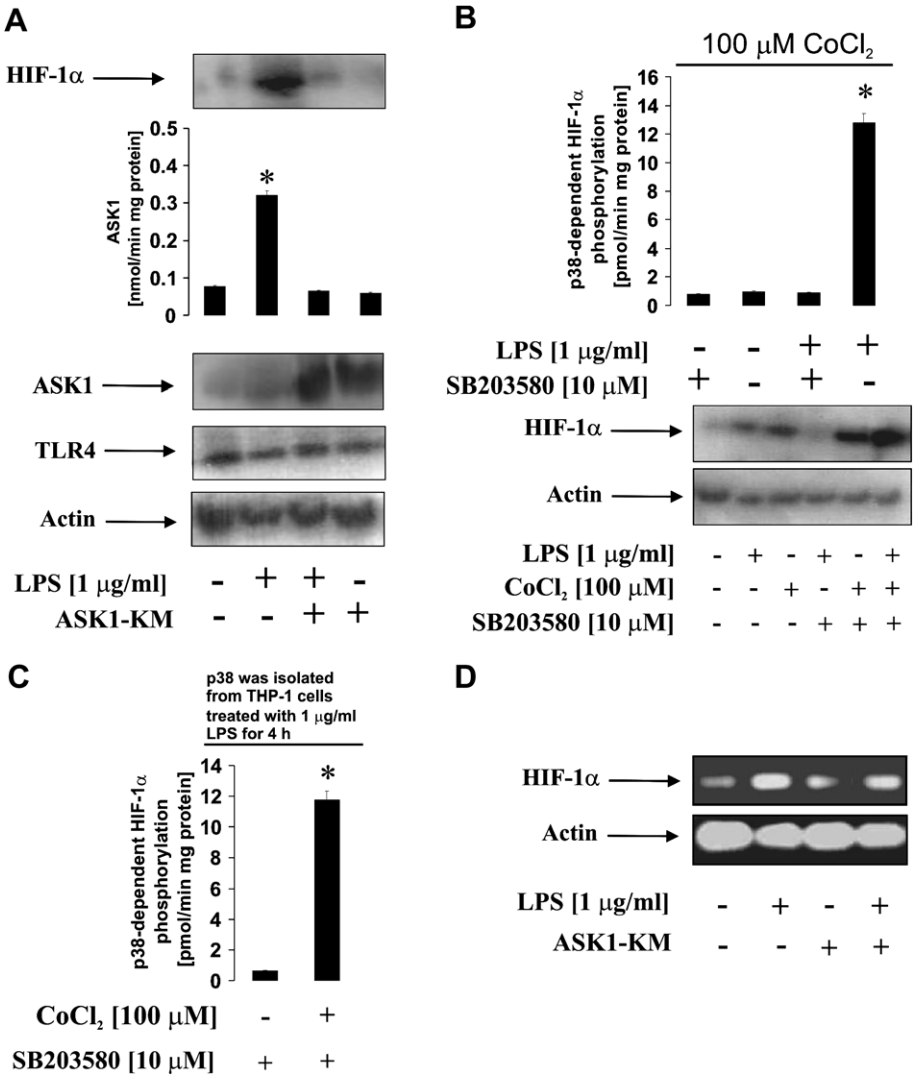

Fig. 4. ASK1 and p38 MAP kinase are critical for LPS-dependent LPS-dependent TLR4-mediated HIF-1α accumulation. (A) THP-1 cells were transiently transfected with ASK1-KM and stimulated for 4 h with LPS. Accumulation of HIF-1α protein, and ASK1 protein stability/kinase activity were analysed. TLR4 level was also monitored. Actin staining was used as a protein loading control (see Figs. 1–3 for the type of assay). (B,C) p38-dependent HIF-1α phosphorylation was studied as outlined in Materials and methods. (D) THP-1 cells were transfected with ASK1-KM followed by 16 h of stimulation with 1 μg/ml LPS. mRNA of HIF-1α and actin were detected by RT-PCR as outlined in Section 2. Digital data are mean values ± S.D. of at least four individual experiments. $^{*}P < 0.01$ vs. control ($^{*}P < 0.01$ between two specific experiments). All Western-blot and RT-PCR data are from one experiment representative of three that gave similar results.

accumulation we transfected THP-1 cells with 2.5 μg/ml dominant-negative form of ASK1 (ASK1-KM) and exposed the cells to 1 μg/ml LPS during 4 h. The increase in amount of ASK1 protein was rather significant and decreased its kinase activity (Fig. 4A). The decrease in HIF-1α accumulation was clear in the cells transfected with ASK1-KM and then stimulated with LPS (Fig. 4A). TLR4 amount in THP-1 cells was not affected by treatments. These data suggest critical role of ASK1 in accumulation of HIF-1α protein in TLR4 signalling. The contribution of ASK1 is most likely in activation of p38, which is important for HIF-1α protein accumulation at some stages. To confirm LPS-dependent p38-mediated HIF-1α protein phosphorylation we have monitored this process as outlined in Section 2. It was clear that in LPS-stimulated cells HIF-1α phosphorylating activity of p38 was quite strong (Fig. 4B).

Furthermore we have isolated p38 from THP-1 cells stimulated with 1 μg/ml LPS and detected that it could phosphorylate HIF-1α protein isolated from THP-1 cells treated with 100 μM cobalt chloride (see Section 2.8 for experimental details, Fig. 3). Finally, we have found that in the amount of HIF-1α mRNA produced in THP-1 cells was increased in the cells exposed to 1 μg/ml LPS. However, when the cells were transfected with ASK1-KM and stimulated with 1 μg/ml LPS, the level of HIF-1α mRNA was lower than the one produced in non-transfected cells stimulated with 1 μg/ml LPS (Fig. 4 D) but no significant reduction was observed. Therefore, ASK1 downstream pathway is most likely important to stabilise HIF-1α protein at the translational/post-translational level. Overall, these data suggest a strong contribution of ASK1 to LPS-dependent accumulation of HIF-1α protein as well as its participation in HIF-1α gene expression.

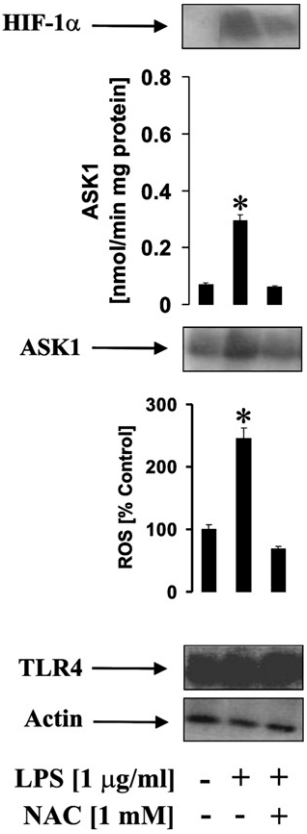

Fig. 5. ROS contribute to LPS-dependent activation of HIF-1α/ASK1 and RNS down-regulate ASK1 kinase activity by direct *S*-nitrosation of the enzyme. THP-1 cells were pre-treated during 1 h with 1 mM NAC and then stimulated during 4 h or 64 h with 1 μg/ml LPS. Accumulation of HIF-1α protein, ASK1 protein stability/kinase activity, Nox-dependent ROS and production were analysed as described in Section 2 (see also Figs. 1–3 for the type of assay). Digital data are mean values ± S.D. of at least four individual experiments. $^{*}P < 0.01$ vs. control. All Western-blot data are from one experiment representative of three that gave similar results.

To verify critical role of ROS in HIF-1α protein accumulation and activation of ASK1 we pre-treated the cells with 1 mM NAC followed by 4 h of stimulation with 1 μg/ml LPS. NAC decreased ROS production and attenuated HIF-1α accumulation/activation of ASK1 but not the amount of TLR4 produced by THP-1 cells (Fig. 5). These data suggest crucial role of Nox-derived ROS for both HIF-1α protein accumulation and activation of ASK1.

Based on the data obtained one could suggest that LPS induces both HIF-1α accumulation and expression as a part of TLR4 signalling. TLR4 associated tyrosine kinases activate PI-specific PLC-1γ which leads to the release of DAG/Ca^{2+} that activate PKCα/β, known to induce Nox activity. Nox generates ROS that contribute to stabilisation but not expression of HIF-1α protein and activate ASK1. ASK1 indirectly activates p38, which also contributes to HIF-1α accumulation. In addition to our conclusions, one could speculate that HIF-1α protein could bring certain contribution to down-regulation of ASK1 as the gene of ASK1 inactivating protein phosphatase 5 has hypoxia responsive elements and is up-regulated by HIF-1 transcription complex [29]. However, this speculation is still a subject for experimental confirmation.

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