Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Hypoxic stress up-regulates the expression of Toll-like receptor 4 in macrophages via hypoxia-inducible factor

So Young Kim,[1,2,3] Yong Jun Choi,[1,2,3] Sun Myung Joung,[1,2,3] Byung Ho Lee,[4] Yi-Sook Jung[5,6] and Joo Young Lee[1,2,3]

[1]*Cell Dynamics Research Centre, Gwangju Institute of Science and Technology, Gwangju,* [2]*Research Centre for Biomolecular Nanotechnology, Gwangju Institute of Science and Technology, Gwangju,* [3]*Department of Life Science, Gwangju Institute of Science and Technology, Gwangju,* [4]*Korea Research Institute of Chemical Technology, Daejon,* [5]*Department of Physiology, Ajou University, Suwon, and* [6]*Brain Korea 21 for Molecular Science and Technology, Ajou University, Suwon, Korea*

doi:10.1111/j.1365-2567.2009.03203.x
Received 17 June 2009; revised 21 October 2009; accepted 21 October 2009.
Correspondence: J. Y. Lee, Department of Life Science, Gwangju Institute of Science and Technology, Gwangju, Korea 500-712.
Email: joolee@gist.ac.kr
Senior author: Joo Young Lee

Summary

Toll-like receptors (TLRs) are germline-encoded innate immune receptors that recognize invading micro-organisms and induce immune and inflammatory responses. Deregulation of TLRs is known to be closely linked to various immune disorders and inflammatory diseases. Cells at sites of inflammation are exposed to hypoxic stress, which further aggravates inflammatory processes. We have examined if hypoxic stress modulates the TLR activity of macrophages. Hypoxia and $CoCl_2$ (a hypoxia mimetic) enhanced the expression of TLR4 messenger RNA and protein in macrophages (RAW264.7 cells), whereas the messenger RNA of other TLRs was not increased. To determine the underlying mechanism, we investigated the role of hypoxia-inducible factor 1 (HIF-1) in the regulation of TLR4 expression. Knockdown of HIF-1α expression by small interfering RNA inhibited hypoxia-induced and $CoCl_2$-induced TLR4 expression in macrophages, while over-expression of HIF-1α potentiated TLR4 expression. Chromatin immunoprecipitation assays revealed that HIF-1α binds to the TLR4 promoter region under hypoxic conditions. In addition, deletion or mutation of a putative HIF-1-binding motif in the TLR4 promoter greatly attenuated HIF-1α-induced TLR4 promoter reporter expression. Up-regulation of TLR4 expression by hypoxic stress enhanced the response of macrophages to lipopolysaccharide, resulting in increased expression of cyclooxygenase-2, interleukin-6, regulated on activation normal T cell expressed and secreted, and interferon-inducible protein-10. These results demonstrate that TLR4 expression in macrophages is up-regulated via HIF-1 in response to hypoxic stress, suggesting that hypoxic stress at sites of inflammation enhances susceptibility to subsequent infection and inflammatory signals by up-regulating TLR4.

Keywords: inflammation; innate immunity; macrophages/monocytes; Toll receptors/Toll-like receptors; transcription factors/gene regulation

Introduction

Toll-like receptors (TLRs) are pattern recognition receptors that detect infectious microbial components and mount immune responses.[1] They can also respond to endogenous host molecules derived from damaged tissue as part of the tissue repair process.[2] They are type I transmembrane receptors with extracellular leucine-rich repeat motifs, a transmembrane domain and an intracellular Toll/interleukin-1 receptor homology domain. To date, at least 13 members of the TLR family, with different ligand specificities, have been identified. The TLRs are mainly expressed in immune cells, including monocytes, macrophages, dendritic cells and B cells, and have critical roles in regulating immune and inflammatory responses. It is now well established that they are intimately involved in the development and progression of various inflammatory diseases, including atherosclerosis, rheumatoid arthritis and cancer.[3–5]

Hypoxia plays an important role in regulating a variety of physiological responses as well as in pathological situations.[6] At sites of inflammation, tissue oxygen levels decrease and generate hypoxic stress, which further aggravates and accelerates inflammation and tissue damage.

Hypoxia-inducible factor 1 (HIF-1) is a major transcription factor activated during hypoxia that plays critical roles in inducing hypoxia-related gene expression and cellular responses.[7,8] It is a heterodimeric protein that consists of HIF-1β and HIF-1α.[9] While HIF-1β is constitutively expressed, HIF-1α is tightly regulated by oxygen. Therefore, the overall activity of HIF-1 is dictated by the intracellular HIF-1α level. Oxygen-activated prolyl hydroxylases hydroxylate HIF-1α at proline residues (402 and 564) within an oxygen-dependent degradation domain. This hydroxylation drives HIF-1α toward ubiquitylation by E3 ubiquitin protein ligase, which is part of the von Hippel–Lindau tumour suppressor protein complex, resulting in the rapid degradation of ubiquitylated HIF-1α.[10,11] Under hypoxic conditions, the enzymatic activity of prolyl hydroxylases is significantly reduced because oxygen is a necessary substrate for its activity. As a result, HIF-1α accumulates and HIF-1 activity is upregulated. It has been recently proposed that hypoxia is an inflammatogen, as it can induce the activation of macrophages, which are major cellular sources of inflammatory mediators in the immune system.[12,13]

Toll-like receptor 4 is reported to be associated with a risk of hypoxia-related diseases, and its expression is upregulated in the tissues of patients with myocardial ischaemia–reperfusion injury.[14] The signs of inflammatory responses and pathological changes upon ischaemic injury in various tissues, including heart, kidney, brain and lung, are significantly decreased in TLR4-deficient mice.[15–18] Hypoxia increases TLR4 expression in microglial cells and lowers it in endothelial cells.[19,20] These findings suggest that controlling TLR4 activity could be a useful strategy for decreasing inflammatory responses aggravated by hypoxic stress. However, the mechanism of which hypoxic stress affects TLR4 activation is not fully understood.

We have investigated the mechanism underlying the regulation of TLR4 by hypoxic stress in immune cells. We found that the hypoxia-induced increase in expression of TLR4 in macrophages is mediated at least in part via HIF-1. Our observations also suggest that the increased expression of TLR4 in macrophages under hypoxic stress enhances the susceptibility of the immune system to subsequent infection and inflammatory signals. Regulation of TLR4 expression may therefore represent a novel therapeutic target in a number of pathological states that can be induced by hypoxia.

Materials and methods

Reagents and plasmids

Purified lipopolysaccharide (LPS) from *Escherichia coli* was obtained from List Biological Laboratories Inc. (Campbell, CA). Cobalt chloride ($CoCl_2$) was purchased from Sigma-Aldrich (St Louis, MO) and dissolved in endotoxin-free water. Antibody against cyclooxygenase-2 (COX-2) was purchased from Cayman (Ann Arbor, MI). Antibody against actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma-Aldrich, unless otherwise stated. The HIF-1α expression plasmid was a gift from Dr L. Eric Huang (University of Utah School of Medicine, UT). Luciferase reporter plasmids containing the TLR4 promoter (− 102/+ 223 and − 518/+ 223) were obtained from Dr Thierry Roger (University of Lausanne, Switzerland). A mutated TLR4 promoter luciferase reporter plasmid (− 518/+ 223) with CGTG changed to AAAG (− 407 to − 404) was prepared by site-directed mutagenesis and confirmed by DNA sequencing. The heat-shock protein 70-galactosidase reporter plasmid was from Dr Robert Modlin (University of California, Los Angeles, CA). All DNA constructs were prepared on a large scale using an EndoFree plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

Cell culture

RAW264.7 murine macrophage cells (ATCC TIB-71) were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (volume/volume) heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone). They were maintained at 37° in a 5% CO_2/air environment. To generate hypoxic stress conditions, cells were placed in an air-tight chamber with inflow and outflow valves infused with a gas mixture (5% CO_2, 10% H_2, 85% N_2) maintaining a low oxygen tension (< 1%), or treated with 100 μM $CoCl_2$, which has been widely used to induce hypoxic stress.[9,21]

Reverse transcription–polymerase chain reaction analysis

Total RNAs were extracted with Welprep™ reagent (Jeil Biotechservice Inc., Daegu, Korea). The RNAs were reverse-transcribed with UmProm-II™ Reverse Transcriptase (Promega, Madison, WI) and amplified with an IQ™5 (Bio-Rad, Hercules, CA) using *Taq* DNA polymerase (Solgent, Daejeon, Korea) for routine polymerase chain reaction (PCR), and IQ™ SYBR® Green Supermix (Bio-Rad) for quantitative real-time PCR. The primers were: *Tlr2*, 5′-GACAAA GCGTCAAATCTCAG-3′ and 5′-CCAGAAGCATCACATGACAG-3′; *Tlr3*, 5′-ATGATGCAGTCTTTCCAGAG-3′ and 5′-GAACACCCTTTCATGATTCAG-3′; *Tlr4*, 5′-CCCTGCATAGAGGTAGTTCC-3′ and 5′-AGCTCAGATCTATGTTCTTGGT-3′; *Tlr5*, 5′-CAGGATGTTGGCTGGTTTCT-3′ and 5′-CGGATAAAGCGTGGAGAGTT-3′; *Tlr7*, 5′-CAAGAAAGATGTCCTTGGCTC-3′ and 5′-TAGGAAACCATCGAAACCCA-3′; *Tlr9*, 5′-GAAAGCATCAACCACACCAA-3′ and 5′-ACAAGTCCACAAAGCGAAGG-3′;

Cox-2, 5′-CATATTTGATTGACAGTCCACC-3′ and 5′-CCTTATTTCCCTTCACACC-3′; *Il-6*, 5′-TTCCTCTCTGCAAGAGACT-3′ and 5′-TGTAT CTCTCTGAAGGACT-3′; *Rantes*, 5′-GCCCACGTCAAGGAGTATTTCTAC-3′ and 5′-AGGACTAGAGCAAGCGATGACAG -3′; *Ip-10*, 5′-TCCTGCTGGGTCTGAGTG-3′ and 5′-ATTCTTGATGGTCTTAGATTCCG-3′; *β-actin*, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ and 5′-TTGCGGTGCACGATGGAGGGGCCGGA-3′. The specificity of the amplified PCR products was assessed by a melting curve analysis. Fold inductions of gene expression were calculated as previously described.[22]

Flow cytometric analysis

RAW264.7 cells were labelled with mouse phycoerythrin (PE)-conjugated anti-mouse TLR4 antibody and mouse PE-conjugated isotype immunoglobulin G1κ (IgG1κ) antibody as an isotype control (eBioscience, San Diego, CA). All staining procedures were performed at 4° in Pharmingen Stain Buffer (BD Pharmingen, San Diego, CA). Cells were analysed by flow cytometry using an EPICS XL-MCL (Beckman Coulter, Fullerton, CA).

Immunostaining and confocal imaging analysis

RAW264.7 cells were grown on glass coverslips (18-mm diameter; Fisher Scientific, Pittsburgh, PA). The cells were labelled with Vybrant™ 1,1′-dioctadecyl–3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) cell-labelling solution (Molecular Probes Inc., Eugene, OR), fixed with paraformaldehyde, washed three times with phosphate-buffered saline, and blocked with 1% bovine serum albumin for 30 min. The cells were then incubated with anti-TLR4 antibody (H-80; Santa Cruz Biotechnology) in blocking buffer overnight at 4° followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich), and mounted with anti-fade solution (Molecular Probes Inc.). The slides were examined with an FV 1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) equipped with 100 × objectives. The images were obtained with FLUOVIEW software (Olympus Corporation, Tokyo, Japan).

RNA interference

Transfection of small interfering RNA (siRNA) into RAW264.7 cells was performed with RNAiMax™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Mouse-specific HIF-1α siRNA and non-targeting control siRNA were synthesized and purified by Bioneer Co. (Daejeon, Korea). The sequences were as follows: HIF-1α siRNA, 5′-AAGCAUUUCUCUCAUUUCCUCAUGG-3′; control siRNA, 5′-GACUACUGGUCGUUGAACU-3′.

Transfection and luciferase assay

RAW264.7 cells were transfected with a luciferase plasmid and a HIF-1α expression plasmid using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. A heat-shock protein 70–β-galactosidase plasmid was co-transfected as an internal control. Total amounts of transfected plasmids were equalized by supplementing with the corresponding empty vector. Luciferase and β-galactosidase enzyme activities were determined using the Luciferase Assay System and β-galactosidase Enzyme System (Promega). Luciferase activity was normalized by β-galactosidase activity.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). RAW264.7 cells were treated with 100 μM $CoCl_2$ for 8 hr. Cells were fixed with formaldehyde, lysed and sonicated to obtain DNA fragments of 200 to 1000 base pairs. Chromatin was then immunoprecipitated with anti-HIF-1α antibody (Novus Biology, Littleton, CO) or rabbit IgG overnight at 4°. Protein G agarose (Upstate Biotechnology) was added and incubated for 1 hr at 4°. The PCR was performed with TLR4 promoter-specific or vascular endothelial growth factor (VEGF) promoter-specific primers spanning the putative HIF-1 binding site. The primers for the TLR4 promoter were 5′-TGAGAACTGCAGAAGGCACTCAA-3′ and 5′-AACTGGCGTGGAGCAACATCCTC-3′, yielding a 357-base-pair product. The VEGF promoter-specific primers spanning the HIF-1 binding site were 5′-TGAATCACCATGCCGGCCTGG-3′ and 5′- AGCTAGGGCCCTGGGGTGAAT-3′ yielding a 333-base-pair product. As a loading control, PCR was carried out directly with input DNA purified from chromatin before immunoprecipitation. Quantitative real-time PCR analysis was performed to determine fold induction of HIF-1α binding to each promoter. A ΔCt_2 value was calculated for each sample by subtracting the Ct value for the input (total DNA) from the Ct value obtained from samples immunoprecipitated with specific antibody. A ΔCt_1 value was calculated by subtracting the Ct value for the input (total DNA) from the Ct value for samples immunoprecipitated with rabbit IgG as a control. ΔΔCt was obtained by subtracting ΔCt_2 from ΔCt_1. Fold differences were calculated using the formula, $2^{\Delta\Delta Ct}$ because one cycle difference is equivalent to a two-fold difference in amplification.

Immunoblotting

Solubilized proteins were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The

membranes were immunoblotted with the indicated primary antibody, followed by secondary antibody coupled to horseradish peroxidase. The reactive bands were visualized with an enhanced chemiluminescence system (Amersham Bioscience, Piscataway, NJ).

Enzyme-linked immunosorbent assays

Protein levels of interleukin-6 (IL-6), regulated on activation normal T cell expressed and secreted (RANTES), and interferon-inducible protein-10 (IP-10) were determined using an enzyme-linked immunosorbent assay kit for each cytokine according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The concentration ranges for the standard curves were from 7·8 to 500 pg/ml for IL-6 and RANTES and from 31·2 to 2000 pg/ml for IP-10. The mean minimum detectable dose of IL-6 and IP-10 was 1·6 and 2·2 pg/ml, respectively. The minimum detectable dose of RANTES was typically < 2 pg/ml.

Statistical analysis

Data are expressed as means ± SEM. Comparisons of data between groups were made using Student's *t*-test. Values of $P < 0{\cdot}05$ were considered significant.

Results

Hypoxic stress induces expression of TLR4 in macrophages

To investigate the effect of hypoxic stress on the expression of TLRs in macrophages, RAW264.7 cells were exposed to hypoxia or treated with $CoCl_2$ (a mimetic of hypoxia). Both hypoxia and $CoCl_2$ increased the levels of TLR4 messenger RNA (mRNA) in a time-dependent manner whereas mRNA levels of other TLRs were not enhanced (Fig. 1a,b). Instead, the level of TLR9 tended to decrease in response to hypoxia or $CoCl_2$. TLR4 protein expression was also enhanced by hypoxia and $CoCl_2$ (Fig. 1c,d).

Hypoxic stress-induced TLR4 expression is mediated through HIF-1

To investigate the mechanism by which TLR4 expression is enhanced by hypoxic stress, we tested if HIF-1 was involved, using siRNA. HIF-1 is a major transcription factor regulating hypoxia-mediated gene expression and cellular responses.[7] Up-regulation of TLR4 mRNA induced by hypoxia or $CoCl_2$ was significantly attenuated when HIF-1α expression was knocked down by siRNA transfection (Fig. 2a). Consistent with this, the HIF-1α siRNA also decreased TLR4 protein expression in response to $CoCl_2$ (Fig. 2b). The siRNA targeting HIF-1α reduced the level of HIF-1α mRNA, but not HIF-2α mRNA, indicating the gene specificity (Fig. S1). To confirm the involvement of HIF-1 in TLR4 expression, we took a gain-of-function approach by over-expressing HIF-1α using HIF-1α plasmid. Transfection of HIF-1α expression plasmid resulted in the increased protein level of nuclear translocated HIF-1α in cells under hypoxic conditions (lane 4 in Fig. S2) compared with mock-transfected cells under hypoxic conditions (lane 2 in Fig. S2). Over-expression of HIF-1α increased luciferase reporter expression driven by the TLR4 promoter, as well as TLR4 mRNA levels, in macrophages treated with $CoCl_2$ (Fig. 3a,b). In contrast, HIF-1α over-expression in macrophages in the absence of $CoCl_2$ exposure did not enhance reporter expression and TLR4 transcript levels, probably because of the rapid degradation of HIF-1α in normoxic conditions (Fig. 3a,b). Consistently, nuclear translocation of HIF-1α was observed only when cells were treated with $CoCl_2$ (Fig. S2). These observations suggest that HIF-1α contributes to TLR4 expression under hypoxic conditions in which the level of HIF-1α protein is maintained.

To see if HIF-1 binds to the TLR4 promoter, we performed ChIP assays in macrophages treated with $CoCl_2$, using a specific antibody against HIF-1α. We present the result of ChIP assays using the VEGF promoter as a positive control because VEGF expression is known to be regulated by HIF-1.[23] The ChIP assays followed by PCR were performed with specific primers for the TLR4 or VEGF promoter and revealed that HIF-1α bound to the TLR4 promoter as well as the VEGF promoter (Fig. 3c). These results showed that HIF-1 can transcriptionally regulate TLR4 expression under hypoxic conditions by direct binding to the TLR4 promoter region. The consensus sequence of the hypoxia-responsive element (HRE) is 5′-CGTG-3′,[9] and the proximal region of the mouse TLR4 promoter has a putative HRE located at − 407 to − 404 (Fig. 3d). To determine if the putative HRE is important for TLR4 expression under hypoxic stress, we used a TLR4 promoter construct mutated in the putative HRE (CGTG to AAAG at − 407 to − 404) and a deletion mutant that contains the TLR4 promoter region up to − 102, in addition to a wild-type TLR4 promoter construct. While luciferase reporter expression was induced by HIF-1α in macrophages transfected with the wild-type construct, this effect was greatly diminished when the putative HRE was mutated or deleted (Fig. 3d). This indicates that the putative HRE located at − 407 to − 404 is involved in HIF-1-mediated TLR4 expression.

Macrophages exposed to hypoxic stress show enhanced responsiveness to LPS

To address the effect of enhanced TLR4 expression under hypoxic stress, we investigated if the responsiveness of macrophages to a TLR4 agonist was increased when cells

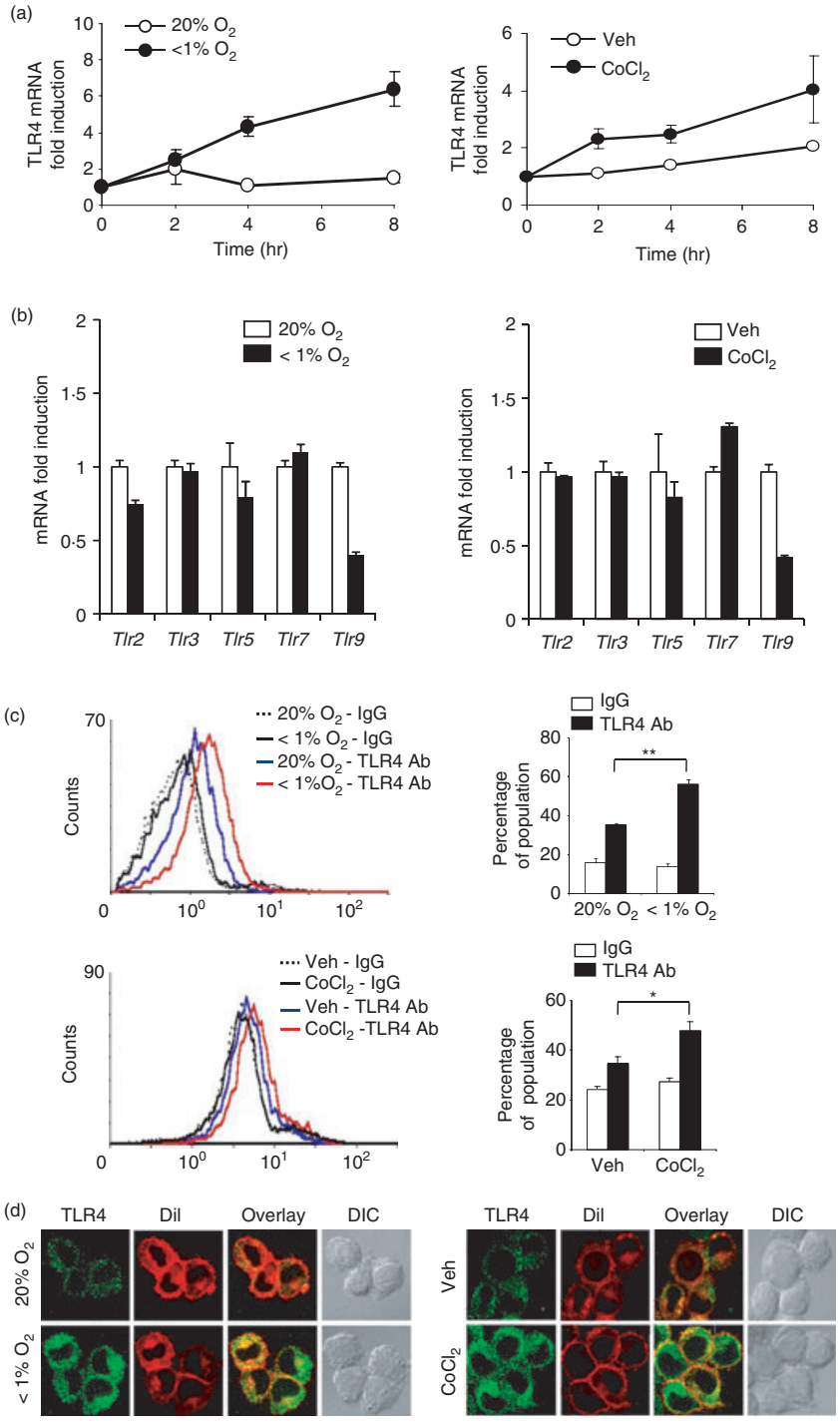


Figure 1. Hypoxic stress increases Toll-like receptor 4 messenger RNA (TLR4 mRNA) and protein expression in macrophages. RAW264.7 cells were incubated under normoxia (20% O_2) or hypoxia (< 1% O_2) or treated with $CoCl_2$ (100 μM) for (a) the indicated times or (b–d) for 8 hr. (a, b) Messenger RNA levels of TLRs were determined by quantitative real-time polymerase chain reaction analysis and normalized with *β*-actin expression. (c) Surface protein expression of TLR4 was analysed by flow cytometry after cells were stained with phycoerythrin-conjugated anti-TLR4 antibody (TLR4 Ab) or isotype control antibody (immunoglobulin G; IgG). (d) Protein expression of TLR4 was analysed by immunostaining and confocal microscopy using anti-TLR4 antibody together with fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody, and fluorescent membrane marker (DiI: 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate). Values are means ± SEM (n = 3–5). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$. Veh, vehicle.

were exposed to hypoxic stress. After RAW264.7 cells had been exposed to hypoxia for 8 hr to increase TLR4 expression, the cells were stimulated with a TLR4 agonist (LPS) under normoxic conditions to avoid a possible direct effect of hypoxia on the parameters examined. Both mRNA and protein levels of COX-2, IL-6 and RANTES induced by LPS were found to be potentiated by pre-exposure to hypoxia or $CoCl_2$ (Fig. 4a–h). The IP-10 mRNA induced by LPS was also greatly enhanced (Fig. 4i). While we could observe the increase in protein levels of COX-2, IL-6 and RANTES by LPS treatment for 8 hr in macrophages, the protein level of IP-10 was not increased by LPS treatment (1 ng/ml) for 8 hr in macrophages (vehicle versus LPS, 170 ± 5·5 versus 160 ± 3·9 pg/ml). Evidently hypoxic stress augments the responsiveness of macrophages to bacterial components

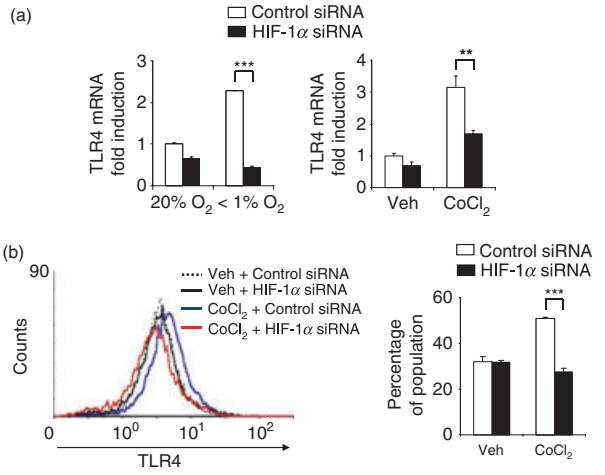
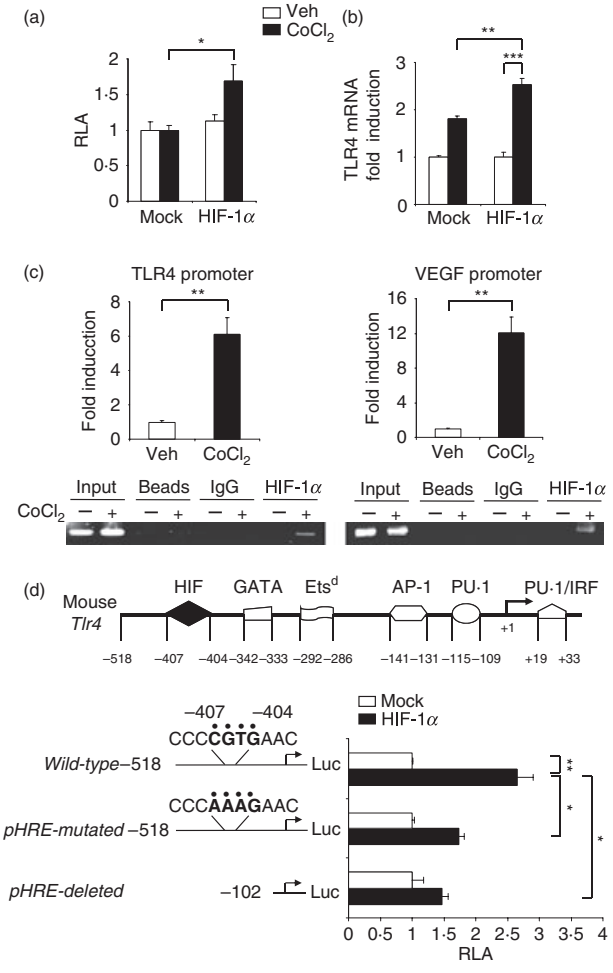


Figure 2. Knockdown of hypoxia-inducible factor 1α (HIF-1α) leads to attenuation of hypoxic stress-induced Toll-like receptor 4 (TLR4) expression. RAW264.7 cells transfected with either small interfering RNA (siRNA) targeting HIF-1α or control siRNA were exposed to normoxia (20% O_2) or hypoxia (< 1% O_2) or treated with $CoCl_2$ (100 μM) for 8 hr. (a) TLR4 messenger RNA levels were determined by quantitative real-time polymerase chain reaction analysis. (b) TLR4 expression was analysed by flow cytometry. Values are means ± SEM (n = 3–4). $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. Veh, vehicle.



as a result of the increased TLR4 expression. This suggests that the higher TLR4 levels in macrophages under hypoxic stress contribute to the aggravated inflammatory responses and enhanced cellular susceptibility to subsequent bacterial infection.

Discussion

Expression of TLRs is not static but rather is modulated dynamically in response to pathogens, a variety of cytokines, and environmental stresses. In particular, regulation of TLR4 levels at infected sites can be critical for appropriate immune and inflammatory responses. In infected and inflamed sites, oxygen levels decrease and generate a hypoxic state because of the massive metabolic oxygen consumption by surrounding cells. This results in changes of the signalling pathways and gene expression related to physiological responses as well as to pathological events.[6–8] We have demonstrated that expression of TLR4 was up-regulated in macrophages exposed to hypoxic stress. While up-regulation of TLR4 mRNA in macrophages was observed after exposure to 2 or 4 hr of hypoxic stress in our study, Ishida *et al.*[20] reported that relatively long-term hypoxia for 48–72 hr diminished TLR4 expression as a result of mitochondrial generation of reactive oxygen

Figure 3. Hypoxic stress-induced Toll-like receptor 4 (TLR4) expression is transcriptionally regulated by hypoxia-inducible factor 1α (HIF-1α). (a) RAW264.7 cells were cotransfected with a luciferase construct driven by the TLR4 promoter (− 518/+ 223) and an expression plasmid for HIF-1α, and treated with $CoCl_2$ (100 μM) for 24 hr. Cell lysates were analysed for luciferase activities. Relative luciferase activity (RLA) was calculated after normalization with β-galactosidase activity. (b) RAW264.7 cells were transfected with the HIF-1α expression plasmid and treated with $CoCl_2$ (100 μM) for 8 hr. TLR4 messenger RNA levels were determined by quantitative real-time polymerase chain reaction (PCR) analysis. (c) RAW264.7 cells were incubated with $CoCl_2$ (100 μM) for 8 hr, and chromatin immunoprecipitation (ChIP) assays followed by PCR were performed using HIF-1α antibody and specific primers for the TLR4 promoter or the vascular endothelial growth factor (VEGF) promoter (positive control). Fold induction was determined by quantitative real-time PCR, and is presented in a bar graph. Lower panel shows gel picture of PCR results: Input, PCR with whole cell genomic DNA; Beads, protein G agarose beads alone; IgG, DNA immunoprecipitated with immunoglobulin G; HIF-1α, DNA immunoprecipitated with anti-HIF-1α antibody. (d) (Upper panel) Map of mouse TLR4 promoter region showing binding sites for various transcription factors including a putative binding site for HIF. (Lower panel) RAW264.7 cells were cotransfected with the HIF-1α expression plasmid and luciferase constructs driven by the wild-type TLR4 promoter (− 518/+ 223), a TLR4 promoter mutated at a putative hypoxia-responsive element (pHRE) or a pHRE-deleted promoter (− 102/+ 223), and then incubated with $CoCl_2$ (100 μM) for 24 hr. Values are means ± SEM (n = 3). $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. Veh, vehicle.

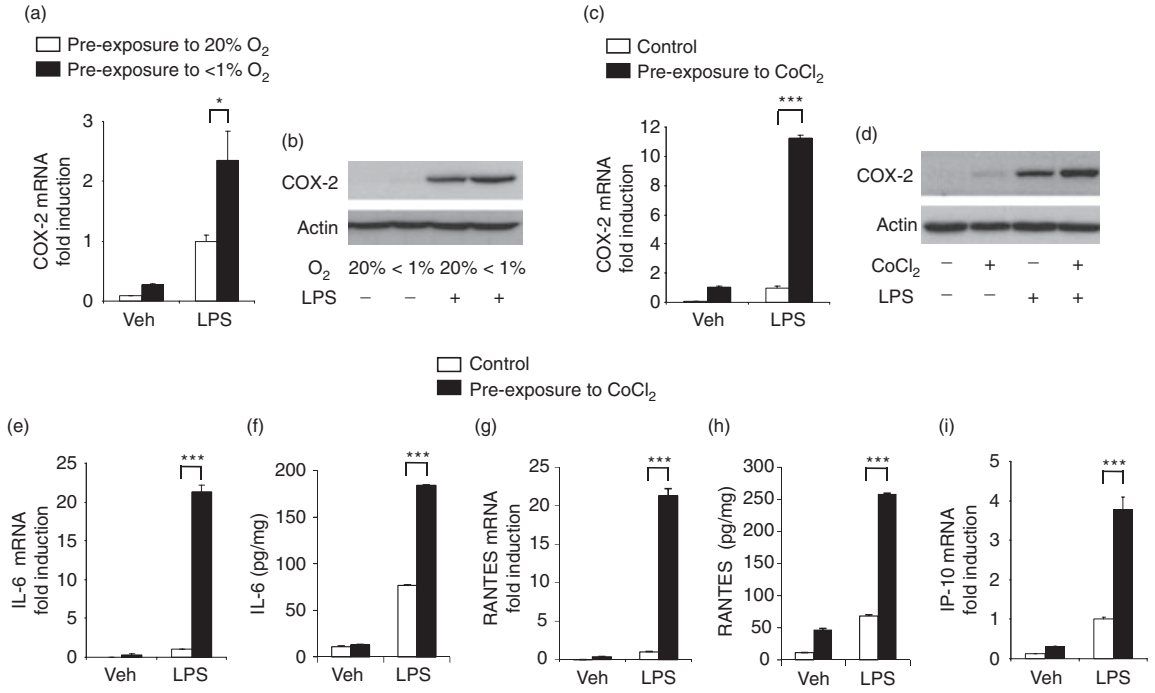


Figure 4. Responsiveness to lipopolysaccharide (LPS) is augmented in macrophages pre-exposed to hypoxic stress. RAW264.7 cells were pre-exposed to normoxia (20% O_2) or hypoxia (< 1% O_2) or treated with $CoCl_2$ (100 μM) for 8 hr. Cells were washed and treated with LPS (a, 5 ng/ml; B, 0·5 ng/ml; c–i, 1 ng/ml) for (a, c, e, g, i) 4 hr or (b, d, f, h) 8 hr under normoxic condition. (a, c, e, g, i) The messenger RNA levels of cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), regulated on activation normal T cell expressed and secreted (RANTES) and interferon-inducible protein-10 (IP-10) were determined by quantitative real-time polymerase chain reaction analysis and normalized by β-actin. (b, d) Cell lysates were analysed for COX-2 and actin by immunoblotting. (f, h) Cell lysates were analysed for IL-6 and RANTES expression by enzyme-linked immunosorbent assay. Values are means ± SEM (n = 3–4). $^*P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$. Veh, vehicle.

species in human umbilical vein endothelial cells. Kuhlicke *et al.*[24] showed that protein expression of TLR2 and TLR6 was increased by relatively long-term hypoxia for 24–72 hr in human microvascular endothelial cells, while the TLR4 mRNA level at 24 hr was not changed by hypoxic stress in bone-marrow-derived dendritic cells. Although differences in the duration of hypoxia and the types of cells may account for differential cellular responses in TLR expression, these results suggest that expression of TLRs such as TLR4 and TLR2 can be dynamically regulated by hypoxic stress. Consistent with our results, many *in vivo* experimental and clinical studies demonstrated that TLR4 expression was elevated in cells and tissues of hypoxia-related disease. The levels of TLR4 mRNA and protein were increased in murine hearts after myocardial ischaemic injury, and in human hearts derived from patients with dilated cardiomyopathy and myocarditis.[14,25] In addition, TLR4 expression was significantly increased in Kupffer cells in rat liver grafts, and in tubular epithelial cells and infiltrating leucocytes within the kidney following ischaemia.[16,26] Expression of TLR4 was observed in murine and human lipid-rich atherosclerotic lesions, while normal aortic tissues showed no or minimal expression.[27] These findings suggest that TLR4 plays critical roles in the pathology of hypoxia-related diseases. Indeed, it has been reported that TLR4 activation is important for inflammatory and pathological responses induced by ischaemic injury in various tissues. Mice that are TLR4-deficient are reported to have minor infarctions and reduced inflammatory responses after brain ischaemic injury.[15] The TLR4-deficient mice were also protected against kidney dysfunction after kidney ischaemic injury, with lowered expression of pro-inflammatory cytokines and chemokines.[16] After lung ischaemia–reperfusion injury, lungs from TLR4-deficient mice showed a marked reduction in vascular permeability, lung myeloperoxidase activity, bronchoalveolar lavage leucocyte accumulation, and bronchoalveolar lavage cytokines/chemokines secretion compared with the lungs of wild-type mice.[17] Deficiency or functional mutation of TLR4 in mice resulted in reduced myocardial infarction size and signs of inflammatory response after myocardial ischaemia–reperfusion injury.[18] Our results also demonstrate that the expression of inflammatory mediators is greatly augmented in macrophages exposed to hypoxic stress because of the elevated level of TLR4 expression in hypoxic states. These findings all indicate that hypoxic stress can aggravate inflammatory responses to infecting bacteria by

up-regulating TLR4 expression in macrophages. They further suggest that TLR4 activity and innate immunity are involved in the organ damage and inflammatory process of ischaemia-related diseases.

It has not been entirely clear which transcriptional factors are involved in the regulation of TLR4 expression under hypoxia. Our results now show that TLR4 expression can be transcriptionally regulated by HIF-1 in macrophages under hypoxic conditions, and our ChIP assays revealed that HIF-1α binds to the TLR4 promoter. Moreover, the proximal promoter region of mouse TLR4 contains a putative HRE (5′-CGTG-3′) located at − 407 to − 404, and the human TLR4 promoter contains at least two putative HREs located at − 2811 to − 2814 and − 1185 to − 1188 according to an analysis of the human *Tlr4* gene (UCSC Genome Browser, accession no. uc004bjz1). Mutation of the CGTG to AAAG, or deletion of the CGTG region, led to a significant reduction of TLR4 promoter reporter activity in response to HIF-1α over-expression, showing that this site is important for HIF-1-mediated TLR4 expression. Over-expression of HIF-1α increased TLR4 mRNA and promoter activity in macrophages under hypoxic conditions, but not under normoxic conditions, possibly because of the rapid degradation of HIF-1α when oxygen is abundant. It has been reported that HIF-1α can be increased by LPS, contributing to LPS-induced sepsis symptomatology and lethality.[28] Together with our results, these suggest that LPS-induced HIF-1α activation and HIF-1α-mediated TLR4 expression can elicit a positive feedback loop to augment macrophage activation. Indeed, hypoxia enhances the phagocytic and microbicidal functions of macrophages.[29] Our results also demonstrate that the responsiveness of macrophages to bacterial components is increased when cells are pre-exposed to hypoxic stress, which possibly leads to enhanced cellular susceptibility to subsequent infection. Rosenberger *et al.* reported that netrin-1 can be induced in an HIF-1α-dependent manner resulting in the attenuation of hypoxia-elicited inflammation. Hypoxia-mediated inflammation was enhanced in *Ntn1*$^{+/-}$ mice as compared with wild-type mice.[30] Netrin-1 may therefore play a role as a feedback inhibitor to attenuate hypoxia-elicited inflammation. Together with our results, these indicate that the activities of pro-inflammatory (TLR4) and anti-inflammatory (netrin-1) mediators can be regulated at the expression level by environmental stress such as hypoxia, leading to the dynamic modulation of susceptibility to infection and subsequent inflammatory and immune responses.

Acknowledgements

This work was supported by a grant from the Cell Dynamics Research Centre (R11-2007-007-02003-0) and by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean Government (MEST) (R01-2007-000-11283-0).

Disclosures

The authors have nothing to disclose.

References

1 Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**:783–801.

2 Rakoff-Nahoum S, Medzhitov R. Role of toll-like receptors in tissue repair and tumorigenesis. *Biochemistry* 2008; **73**:555–61.

3 Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 2006; **6**:823–35.

4 Lee JY, Hwang DH. The modulation of inflammatory gene expression by lipids: mediation through Toll-like receptors. *Mol Cell* 2006; **21**:174–85.

5 O'Neill LA. Therapeutic targeting of Toll-like receptors for inflammatory and infectious diseases. *Curr Opin Pharmacol* 2003; **3**:396–403.

6 Lewis JS, Lee JA, Underwood JC, Harris AL, Lewis CE. Macrophage responses to hypoxia: relevance to disease mechanisms. *J Leukoc Biol* 1999; **66**:889–900.

7 Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; **3**:721–32.

8 Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 2003; **9**:677–84.

9 Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* 1995; **92**:5510–4.

10 Ivan M, Kondo K, Yang H *et al.* HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science* 2001; **292**:464–8.

11 Salceda S, Caro J. Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997; **272**:22642–7.

12 Murdoch C, Muthana M, Lewis CE. Hypoxia regulates macrophage functions in inflammation. *J Immunol* 2005; **175**:6257–63.

13 Karhausen J, Haase VH, Colgan SP. Inflammatory hypoxia: role of hypoxia-inducible factor. *Cell Cycle* 2005; **4**:256–8.

14 Frantz S, Kobzik L, Kim YD, Fukazawa R, Medzhitov R, Lee RT, Kelly RA. Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium. *J Clin Invest* 1999; **104**:271–80.

15 Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. *Circulation* 2007; **115**:1599–608.

16 Wu H, Chen G, Wyburn KR *et al.* TLR4 activation mediates kidney ischemia/reperfusion injury. *J Clin Invest* 2007; **117**:2847–59.

17 Shimamoto A, Pohlman TH, Shomura S, Tarukawa T, Takao M, Shimpo H. Toll-like receptor 4 mediates lung ischemia–reperfusion injury. *Ann Thorac Surg* 2006; **82**:2017–23.

18 Oyama J, Blais C Jr, Liu X, Pu M, Kobzik L, Kelly RA, Bourcier T. Reduced myocardial ischemia–reperfusion injury in toll-like receptor 4-deficient mice. *Circulation* 2004; **109**:784–9.

19 Ock J, Jeong J, Choi WS, Lee WH, Kim SH, Kim IK, Suk K. Regulation of Toll-like receptor 4 expression and its signaling by hypoxia in cultured microglia. *J Neurosci Res* 2007; **85**:1989–95.

20 Ishida I, Kubo H, Suzuki S *et al.* Hypoxia diminishes toll-like receptor 4 expression through reactive oxygen species generated by mitochondria in endothelial cells. *J Immunol* 2002; **169**:2069–75.

21 Epstein AC, Gleadle JM, McNeill LA *et al. C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 2001; **107**:43–54.

22 Lee JY, Zhao L, Youn HS *et al.* Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. *J Biol Chem* 2004; **279**:16971–9.

23 Kimura H, Weisz A, Ogura T *et al.* Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. *J Biol Chem* 2001; **276**:2292–8.

24 Kuhlicke J, Frick JS, Morote-Garcia JC, Rosenberger P, Eltzschig HK. Hypoxia inducible factor (HIF)-1 coordinates induction of Toll-like receptors TLR2 and TLR6 during hypoxia. *PLoS ONE* 2007; **2**:e1364.

25 Satoh M, Nakamura M, Akatsu T, Iwasaka J, Shimoda Y, Segawa I, Hiramori K. Expression of Toll-like receptor 4 is associated with enteroviral replication in human myocarditis. *Clin Sci (Lond)* 2003; **104**:577–84.

26 Peng Y, Gong JP, Liu CA, Li XH, Gan L, Li SB. Expression of toll-like receptor 4 and MD-2 gene and protein in Kupffer cells after ischaemia–reperfusion in rat liver graft. *World J Gastroenterol* 2004; **10**:2890–3.

27 Xu XH, Shah PK, Faure E *et al.* Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation* 2001; **104**:3103–8.

28 Peyssonnaux C, Cejudo-Martin P, Doedens A, Zinkernagel AS, Johnson RS, Nizet V. Cutting edge: essential role of hypoxia inducible factor-1α in development of lipopolysaccharide-induced sepsis. *J Immunol* 2007; **178**:7516–9.

29 Anand RJ, Gribar SC, Li J, Kohler JW, Branca MF, Dubowski T, Sodhi CP, Hackam DJ. Hypoxia causes an increase in phagocytosis by macrophages in a HIF-1α-dependent manner. *J Leukoc Biol* 2007; **82**:1257–65.

30 Rosenberger P, Schwab JM, Mirakaj V, Masekowsky E, Mager A, Morote-Garcia JC, Unertl K, Eltzschig HK. Hypoxia-inducible factor-dependent induction of netrin-1 dampens inflammation caused by hypoxia. *Nat Immunol* 2009; **10**:195–202.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Knockdown of hypoxia-inducible factor 1α (HIF-1α) expression by small interfering RNA transfection.

Figure S2. Expression of hypoxia-inducible factor 1α (HIF-1α) by transfection of HIF-1α expression plasmid.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

