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Hypoxia Diminishes Toll-Like Receptor 4 Expression Through Reactive Oxygen Species Generated by Mitochondria in Endothelial Cells

Itaru Ishida,* Hiroshi Kubo,** Satoshi Suzuki,* Tomoko Suzuki,† Sachiko Akashi,§ Kunihiko Inoue,* Sumiko Maeda,* Hideaki Kikuchi,† Hidetada Sasaki,‡ and Takashi Kondo*

Hypoxia and inflammation often occur simultaneously due to prevention of adequate gas exchange. Understanding the influence of hypoxia on the inflammatory response is important because hypoxia directly regulates expression of many genes, including those regulating inflammation, and plays a role in modulating the resolution of an inflammatory response. LPS is a major mediator of cellular injury and inflammation that induces its effects through Toll-like receptor 4 (TLR4). The aim of this study was to evaluate the effect of hypoxia on TLR4 expression. Hypoxia decreased TLR4 expression on cultured endothelial cells. Furthermore, LPS-induced ICAM-1 up-regulation was decreased by hypoxia. Because reactive oxygen species (ROS) generated from mitochondria are one of the signaling molecules induced by hypoxia, the role of ROS in hypoxia-induced TLR4 down-regulation was evaluated. Our data showed that hypoxia increased ROS generation and that hypoxia-induced TLR4 down-regulation was inhibited by myxothiazol, a mitochondrial site III electron transport inhibitor. Hypoxia also inhibited AP-1 translocation. Since the TLR4 promoter has a binding site for AP-1, hypoxia-induced TLR4 down-regulation may be due to an ROS-mediated decrease in AP-1-binding activity. We conclude that hypoxia decreases TLR4 expression in endothelial cells and that this change is mediated by mitochondrial ROS leading to attenuation of AP-1 transcriptional activity. The Journal of Immunology, 2002, 169: 2069–2075.

Materials and Methods

Cells and reagents
HUVEC, human pulmonary artery endothelial cells (HPAEC), and culture medium HuMedia EB2 were purchased from Kurabo (Osaka, Japan). Anti-human TLR4 mAb (HTA1216) was purified from BALB/c mice immunized with the Ba/F3 line expressing TLR4, as described previously (16). PE-conjugated mouse anti-human ICAM-1 mAb was purchased from BD

CM-H₂DCFDA, 5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; DCF, dichlorodihydrofluorescein.
PharMingen (San Diego, CA). LPS from Escherichia coli 055:B5 was purchased from Difco (Detroit, MI). Myxothiazol was purchased from ICN Biomedicals (Aurora, OH). 5-(and -6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was purchased from Molecular Probes (Eugene, OR).

Cell culture
HUVEC or HPAEC were grown to confluence in HuMedia EB2 medium supplemented with 2% FBS, 10 ng/ml human epidermal growth factor, 1 mg/ml hydrocortisone, 50 mg/ml gentamicin, 50 ng/ml amphotericin B, 5 ng/ml human fibroblast growth factor β, and 10 mg/ml hepatin at 37°C under 5% CO2 in 95% air. The culture medium was changed to HuMedia EB2 without all supplements except 2% FBS before study. The cultures were then placed in a humidified airtight chamber. To expose to hypoxic conditions, the air in the chamber was completely exchanged to a gas mixture containing 5% CO2 and 95% N2. Then cells were incubated at 37°C within the chamber. PO2, PO2, and pH levels in the culture medium were measured periodically. HPAEC were incubated in either this normoxic or hypoxic environment for 48 h, then TLR4 mRNA expression was measured by RT-PCR, described below. HUVEC were incubated in either a normoxic or hypoxic environment for 12, 24, 48, or 72 h. Then the following experiments were performed.

RT-PCR analysis and Northern blot analysis
Total cellular RNA was isolated by RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. CDNAs was synthesized from 2 µg total RNA by extension of random hexamers with Superscript II (Life Technologies, Rockville, MD). PCR of the CDNAs was performed in a final volume of 20 µl containing 1.5 mmol/L MgCl2, 0.5 U of DNA polymerase (AmpliTag; PerkinElmer, Branchburg, NJ), and 0.2 µM specific primers. PCR amplification was performed for 32 cycles at 95°C for 40 s, 54°C for 40 s, and 72°C for 60 s. The oligonucleotide primers used for RT-PCR were: human TLR4, 5’-TGG ATA CGT TTC CTT ATA CTT GGA-3’ and 5’-GAA ATG GAG GCA CCC CTT C-3’; human MD-2, 5’-CTA GAA GCA GTA TGT GGT CT-3’ and 5’-TTA GGT TGG TGT TGG AGG ATG AC-3’; human β-actin, 5’-GCT GGG CTA TCC CTG TAG G-3’ and 5’-TGC CTC AGG AGA CCC CAA-3’. The synthesized PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. PCR bands were scanned and imported to Power Macintosh, and the intensities of each band were analyzed using NIH Image software.

For Northern blot analysis, 20 µg of total RNA was electrophoresed, transferred to a positively charged nylon membrane (Hybond-N+; Amer sham Pharmacia Biotech, Uppsala, Sweden), and hybridized with a 32P-labeled cDNA probe at 65°C. A cDNA probe specific for TLR4 was obtained by RT-PCR using the set of specific primers described above. The intensity of hybridization signals was quantitated by densitometric scanning of autoradiographs using a Fujix BAS2000 Bio-image Analyzer (Fuji, Tokyo, Japan).

Immunostaining for flow cytometry
Surface expression of TLR4 on HUVEC was determined using mouse anti-human TLR4 mAb (BD Pharmingen) and mouse anti-human TNF-R1 mAb (Genzyme/Thechne, Minneapolis, MN). Cells were analyzed by an EPICS XL-MCL (Beckman Coulter, Fullerton, CA).

Measurement of intracellular ROS
Intracellular ROS generation was assessed using CM-H2DCFDA, as described previously (17). HUVEC were seeded on 96-well tissue culture plates. After HUVEC were grown to confluence, cells were incubated at 37°C for 24 h under either normoxic or hypoxic conditions. Then CM-H2DCFDA (200 µM) was added and the incubation was continued for 45 min. CM-H2DCFDA is a compound that enters cells and fluoresces only when it is oxidized by ROS, particularly hydrogen peroxide and hydroxyl radicals. ROS generation was assessed by measuring the dichlorodihydrofluorescein (DCF) fluorescence on Fluoroskan Ascent FL (excitation 490 nm, emission 526 nm; Thermo Labsystems, Helsinki, Finland). To determine whether ROS generated from mitochondria affect TLR4 expression, myxothiazol (1 µM, mitochondrial site III electron transport inhibitor) was added before the incubation to block mitochondrial ROS generation.

EMSA
Dishes were gently scraped, and cells were collected by centrifugation at 300 × g for 5 min. Cells were lysed for 15 min at 4°C in a solution containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.5% Nonident P-40. Nuclei were collected by centrifugation at 1500 × g for 30 s and then suspended in a solution of 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. The mixture was shaken vigorously for 15 min at 4°C, and the supernatant was collected after centrifugation for 5 min at 10,000 × g. Binding reactions were performed with 2 µg of nuclear protein in 20 mM HEPES (pH 7.9), 100 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, 20% glycerol, 2 µg of salmon sperm DNA, 2 mM MgCl2, and 10,000 cpm of 32P-labeled oligonucleotide. DNA complexes were separated on a 4% polyacrylamide gel in TBB buffer (350 mM Tris-HCl, pH 8.9, 100 mM NaCl, 2.5 mM KCl, 50 mM MgCl2) and visualized by autoradiography. Quantitation was performed with a two-dimensional gel scanner (model 9000; Bio-Rad, Hercules, CA). The relative binding activities for the AP-1 binding site (sequence: 5’-CGG TGG ATG AGT CCG GCA-3’) or IRF-1 binding site (5’-CCG TAA GAC TCA TCA CCC TT-3’) was labeled by standard procedures.

Luciferase assays
HUVEC were seeded in 24-well plates at a density of 1 × 105 cells/well 1 day before transfection. An equal amount of luciferase reporter plasmid pPAI-Luc (PathDetect AP-1 cis-Reporting System; Stratagene, La Jolla, CA) was transfected using Lipofectamine (Life Technologies, Rockville, MD) according to the manufacturer’s protocol. After a 48-h incubation under either normoxic or hypoxic conditions, HUVEC were harvested, washed, and lysed in 100 µl of lysis buffer, and luciferase activity was measured using 20 µl of lyase and 100 µl of luciferase substrate (Nippon Gene, Toyama, Japan). The luminescence was quantitated as a relative light unit on a luminometer (Lumat LB 9507; Berthold Japan, Tokyo, Japan). Results are presented as the average of four experiments where background has been subtracted.

ICAM-1 immunostaining for flow cytometry
Either myxothiazol (1 µM) or vehicle was added before the incubation, then HUVEC were incubated for 48 h under either normoxic or hypoxic conditions. After the exposure, HUVEC were incubated with LPS (50 ng/ml) at 37°C for 4 h. Then cells were stained with PE-conjugated mouse anti-human ICAM-1 mAb and analyzed by EPICS XL-MCL.

Statistics
Data were expressed as the mean ± SEM. Data were compared using ANOVA. When overall differences were identified, multiple contrasts with a Bonferroni adjustment were used to identify which groups were significantly different. Statistical significance was defined as p < 0.05.

Results
Hypoxia down-regulates TLR4 expression
To determine whether hypoxia changed TLR4 expression, HUVEC were incubated under hypoxic conditions and TLR4 expression was measured by Northern blot analysis, RT-PCR, and immunostaining. The oxygen pressure of the culture medium was 38.1 ± 2.1 torr under hypoxic conditions and 148.3 ± 1.8 torr in normoxic conditions. There was no significant difference in either pH or PCO2 in the culture media (data not shown). These conditions were reached by 30 min after starting the exposure and were constant throughout the experiment. There was no loss of viability after the exposure (data not shown). TLR4 mRNA expression decreased after hypoxia for 48 h (Fig. 1A). In contrast, hypoxia did not change mRNA expression of MD-2, an adaptor protein required for TLR4-induced signaling (Fig. 1A). This decrease in TLR4 mRNA expression was also observed in HPAEC after hypoxia for 48 h (Fig. 1B). TLR4 mRNA expression decreased to ~70% of the normoxic group in HUVEC (Fig. 1C). This TLR4 mRNA down-regulation occurred in a time-dependent manner (Fig. 1D). Immunostaining and flow cytometry for TLR4 protein expression on the cell surface demonstrated that TLR4 expression decreased to ~60% of the normoxia group by 48 h of exposure to hypoxia. This decrease was not apparent by 24 h (Fig. 2). Additionally, both IL-1R1 and TNF-R1 protein expression on HUVEC were also decreased by hypoxia (Fig. 3).
The role of mitochondrial ROS generation

Recently, mitochondria have been described as a major source of ROS in response to hypoxia, and hypoxia increases ROS generation as a result of electron flux through mitochondrial complex III (18). To determine whether mitochondria-generated ROS play a role in hypoxia-induced TLR4 down-regulation, we measured ROS generation by hypoxia exposure in the presence and absence of myxothiazol, a mitochondrial site III electron transport inhibitor that blocks mitochondrial ROS generation. Hypoxia induced a significant increase in DCF fluorescence within HUVEC (Fig. 4A), indicating that intracellular ROS were generated in HUVEC by hypoxia. Furthermore, myxothiazol abolished the ROS generation induced by hypoxia (Fig. 4A). To further study the importance of mitochondria-generated ROS on the changes in TLR4 expression, myxothiazol was added to the culture medium before exposure to hypoxia.

FIGURE 1. Hypoxia-induced TLR4 mRNA down-regulation in HUVEC. A, A representative gel of RT-PCR products, which was performed with total RNA from HUVEC incubated under either normoxic or hypoxic conditions for 48 h. TLR4 mRNA was decreased after hypoxia exposure while MD-2 mRNA did not change. B, A representative gel of RT-PCR products, which was performed with total RNA from HPAEC incubated under either normoxic or hypoxic conditions for 48 h. TLR4 mRNA was decreased after hypoxia. C, Northern blot analysis showed that TLR4 mRNA was significantly decreased by hypoxia (n = 4; *, p < 0.05). Data are expressed as the percentage of the normoxia group by calculating the intensities of each band. D, Total RNA from HUVEC incubated under either normoxic or hypoxic conditions for 12, 24, and 48 h were reverse transcribed and amplified with TLR4-specific primers. Hypoxia decreased TLR4 mRNA expression in HUVEC in a time-dependent manner (n = 5; *, p < 0.05). There were no significant differences between normoxia and hypoxia at 12- or 24-h incubations (n = 3).

FIGURE 2. Hypoxia decreased TLR4 protein expression on HUVEC. HUVEC were incubated under either normoxic or hypoxic conditions for 12, 24, 48, and 72 h. Then cells were stained with HTA1216 (1 mg/10⁶ cells), visualized by goat anti-mouse IgG-dichlorotriazinyl aminofluorescein, and analyzed by flow cytometry. Data are expressed as a percentage of the expression in normoxic cells by calculating the mean fluorescence intensities. A decrease in TLR4 protein expression occurred in a time-dependent manner (n = 3; *, p < 0.05). At 12- or 24-h incubation, however, there were no significant differences between hypoxic and normoxic conditions.

FIGURE 3. Hypoxia decreased IL-1R1 and TNF-R1 protein expression on HUVEC. HUVEC were incubated under either normoxic or hypoxic conditions for 48 h. Then cells were stained with either anti-human IL-1R1 mAb or anti-human TNF-R1 mAb and analyzed by flow cytometry. Data are expressed as a percentage of the expression in normoxic cells by calculating the mean fluorescence intensities. A, IL-1R1 expression was significantly decreased by hypoxia (n = 4; *, p < 0.05). B, TNF-R1 expression was significantly decreased by hypoxia (n = 4; *, p < 0.05).
either normoxia or hypoxia and TLR4 expression was measured. As seen in Fig. 4, B and C, myxothiazol completely prevented the hypoxia-induced down-regulation of TLR4 at both the mRNA and protein level.

**Nuclear AP-1 and IRF/PU.1 activity**

There are potential binding sites for AP-1 and IRF/PU.1 within the proximal promoter region of TLR4 (12). To determine whether AP-1 or IRF/PU.1 contributes to hypoxia-induced TLR4 down-regulation, the translocation of AP-1 and IRF/PU.1 to nuclei and their ability to bind consensus oligonucleotides were examined by EMSA. Nuclear AP-1 activity using consensus oligonucleotides was significantly decreased by hypoxia (Fig. 5A). AP-1 activity measured using the unique sequence of human TLR4 promoter region (5'-GAG GTC AGA TGA CTA ATT GGG A-3') as the binding oligonucleotide also demonstrated that the AP-1 activity was similarly decreased by hypoxia (data not shown). In contrast, nuclear IRF/PU.1 activities did not change under hypoxic conditions (Fig. 5B). Administration of myxothiazol tended to prevent this decrease in AP-1 activity (Fig. 6, p = 0.054).

**Luciferase assays**

HUVEC transiently transfected with AP-1 reporter plasmid exhibited decreased luciferase activity after hypoxia exposure. Administration of myxothiazol prevented a hypoxia-induced decrease in luciferase activity (Fig. 7).

**LPS-induced ICAM-1 expression**

To determine whether hypoxia-induced down-regulation of TLR4 had an effect on the LPS response in endothelial cells, we examined the changes in LPS-induced ICAM-1 expression. Flow cytometric analysis showed that LPS induced ICAM-1 up-regulation under normoxic conditions (Fig. 8A), but this increase was less under hypoxic conditions (Fig. 8B). PE-fluorescent intensity increased 4.7-fold above the baseline under normoxic conditions but only 2.3-fold under hypoxic conditions. This effect of hypoxia on cellular hyporesponsiveness to LPS was prevented by myxothiazol (Fig. 8D).

**Discussion**

Our observations indicate that hypoxia down-regulates TLR4 mRNA and protein expression in endothelial cells within 48 h, and Fig. 9 illustrates the postulated pathway through which this down-regulation occurs. Other investigators have demonstrated that changes in TLR4 expression directly alter cellular responsiveness to LPS, and LPS tolerance has been clearly associated with the down-regulation of surface TLR4 expression (11). Our data show that the increase in ICAM-1 expression induced by LPS was diminished by hypoxia, indicating that hypoxia-induced down-regulation of TLR4 does alter cellular responsiveness to LPS. Taken together, our data suggest that hypoxia induces alterations in the inflammatory response of endothelial cells to LPS and thus to many Gram-negative pathogens.

The effect of hypoxia appears to be focused directly on TLR4 expression and not on expression of associated proteins that modulate TLR4 signaling. MD-2 is an adapter protein that is associated with TLR4 at the membrane and is required for TLR4-initiated signaling (16). Hypoxia does not alter MD-2 expression in endothelial cells (Fig. 1A). CD14 is another regulator of LPS signaling, but endothelial cells do not express this molecule (19). Thus, the effects of hypoxia on cellular responsiveness to LPS appear to be directly and specifically on TLR4 expression.

The net effect of TLR4 down-regulation to the patient is not clear. Diminished responsiveness to circulating LPS may have important protective effects (20, 21). However, the reduction in TLR4 expression may enhance susceptibility to bacterial infection. A destructive mutation in the TLR4 gene that results in null expression
predisposes mice to develop Gram-negative sepsis (6), suggesting that decreased expression of functional TLR4 on immune cells prevents clearance of Gram-negative bacteria. The effect of hypoxia on TLR4 expression on immune cells is not known. Whether the observed decrease in TLR4 expression on endothelial cells or on other nonimmune cells increases susceptibility of bacterial infection is also not yet clear.

How do endothelial cells sense hypoxic conditions? Our data indicate that mitochondria-generated ROS are involved in this hypoxia-induced TLR4 down-regulation. Recently, several reports showed that hypoxia increases ROS generation from mitochondria and these oxidant signals appear to act as second messengers in adaptive responses to hypoxia. Thus, the mitochondrial respiratory chain is known to be one site of hypoxia sensing (13, 14, 15).

FIGURE 5. Changes in nuclear AP-1 and IRF/PU.1 activity. Nuclear protein was extracted from HUVEC incubated under either normoxic or hypoxic conditions for 48 h. AP-1-binding activity was evaluated by EMSA using the AP-1 consensus oligonucleotide. IRF/PU.1-binding activity was examined by using the unique sequence of human TLR4 promoter region. A, AP-1 activity was significantly decreased under hypoxic conditions (n = 4; *, significantly different from the normoxia group). B, In contrast, IRF/PU.1 activity did not change under hypoxic conditions.

FIGURE 6. Effect of myxothiazol, mitochondrial site III electron transport inhibitor, on AP-1 activity. Myxothiazol (1 μM) was added to the culture medium before the normoxia or hypoxia exposure to block mitochondrial ROS generation. After 48 h, nuclear protein was extracted from HUVEC and then EMSA was performed using the AP-1 consensus oligonucleotide. Myxothiazol tends to inhibit the hypoxia-induced decrease in AP-1 activity (p = 0.054; n = 4; *, significantly different from the normoxia group).

FIGURE 7. Changes in AP-1 reporter activity. The luciferase reporter plasmid pAP1-Luc was transfected in HUVEC, then cells were incubated in either normoxic or hypoxic conditions for 48 h. Myxothiazol (1 μM) or vehicle was added to the culture medium before the normoxia or hypoxia exposure. After 48 h, cells were lysed and luciferase activity was measured. Luciferase activity was expressed as a ratio to the value of the normoxia group (n = 4; *, significantly different from the normoxia group; †, significantly different from the myxothiazol-pretreated HUVEC exposed to hypoxia).

FIGURE 8. Changes in LPS-induced ICAM-1 up-regulation on HUVEC. HUVEC were incubated under either normoxic or hypoxic conditions for 48 h and then challenged with LPS (50 ng/ml) for 4 h. Myxothiazol (1 μM) or vehicle was added to the culture medium before the normoxia or hypoxia exposure. Cells were stained with PE-conjugated mouse anti-human ICAM-1 Ab and analyzed by flow cytometry. A representative histogram of five measurements is shown in each group. A, LPS increased expression of ICAM-1 on HUVEC during normoxia. B, This LPS-induced ICAM-1 up-regulation was diminished in the cells incubated under hypoxic conditions. C, Myxothiazol did not affect LPS-induced ICAM-1 up-regulation under normoxic conditions. D, Myxothiazol restored the responsiveness to LPS under hypoxic conditions.
data show that hypoxia induces ROS generation in HUVEC. Myxothiazol, a mitochondrial site III electron transport inhibitor, attenuated the hypoxia-induced ROS generation (Fig. 4A), suggesting that mitochondria-generated ROS play a role in hypoxia sensing in endothelial cells. Furthermore, myxothiazol completely prevented the hypoxia-induced TLR4 down-regulation (Fig. 4, B and C). Taken together, mitochondria-generated ROS are required for hypoxia-induced TLR4 regulation.

It is not clear how mitochondria-generated ROS transduce the signal for TLR4 down-regulation. Potential binding sites for AP-1 and IRF/PU.1 are present in the promoter region of the human TLR4 gene, and IRF/PU.1 participate in the basal regulation of human TLR4 in myeloid cells by deletion analysis of the TLR4 promoter (12). Additionally, IFN-γ up-regulates TLR4 expression in HUVEC (22). These data suggest that IFN-γ and IRF/PU.1 contribute to the TLR4 up-regulation. However, IFN-γ is not detectable in HUCVEC culture (23) and participation of IRF in this TLR4 down-regulation in HUVEC seems unlikely. Interestingly, our data indicate that hypoxia decreases AP-1 but not IRF/PU.1 activity, suggesting that AP-1 but not IRF/PU.1 plays a major role in TLR4 down-regulation. How hypoxia attenuates the AP-1-binding activity is not clear. Oxidation of a conserved cysteine residue in the DNA-binding domains of the AP-1 protein decreases DNA binding and transactivation of AP-1 (24, 25). Thus, oxidation of AP-1 by hypoxia-induced ROS is the most likely cause of decreased AP-1 activity, although alterations in the translocation of AP-1 cannot be excluded. We therefore conclude that a decrease in AP-1-binding activity by hypoxia may contribute to the hypoxia-induced TLR4 down-regulation (Fig. 9).

Hypoxia also diminished the receptor expression of inflammatory cytokines, IL-1R1 and TNF-R1 (Fig. 7). Although both IL-1R1 and TNF-R1 have an AP-1 binding site in their promoter region (26, 27), interestingly IL-1R1, which initiates the same intracellular signaling pathway as TLR4, showed a similar degree of down-regulation in TLR4 expression after hypoxia exposure. Therefore, to understand the net effect of hypoxia on the whole inflammatory process and intracellular signaling pathways is an interesting next question.

In summary, this study demonstrates that TLR4 expression on HUVEC was down-regulated by hypoxia at both the mRNA and protein levels. This decrease was mediated by ROS generated from mitochondria. These ROS may be acting by decreasing the AP-1-binding activity. This decrease in TLR4 expression was associated with a decrease in ICAM-1 up-regulation induced by LPS, suggesting that the reduction in TLR4 expression has important physiologic sequelae. Our data suggesting that hypoxia induces hyporesponsiveness to LPS lead to important questions about the effects of hypoxia in patients with sepsis or endotoxemia. Since both LPS and hypoxia are potent inducers of cellular apoptosis (28, 29), the decrease in responsiveness to LPS under hypoxic conditions may have a cell-protective effect.

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