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Retinoic Acid-Inducible Gene-I Is Induced in Endothelial Cells by LPS and Regulates Expression of COX-2

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Bacterial lipopolysaccharides (LPS) induce expression of multiple genes in endothelial cells, which are critical cellular effectors in various pathologic syndromes. Using subtractive hybridization to identify genes that are differentially induced in human endothelial cells treated with LPS, we found that retinoic acid-inducible gene I (RIG-I) is induced in endothelial cells stimulated with LPS. RIG-I encodes a protein belonging to the DExH-box family which has diverse roles in regulation of gene expression and cellular functions. Cyclooxygenase-2 (COX-2) is also induced in endothelial cells by LPS. Overexpression of RIG-I selectively upregulated expression of COX-2 and also induced COX-2 promoter activity. RIG-I is an inducible gene in stimulated endothelial cells that may have important roles in vascular pathology by virtue of its ability to regulate expression of the COX-2 gene product.

Key Words: **RIG-I; LPS; COX-2; endothelial cells.**

Vascular endothelial cells play critical roles in acute and chronic inflammation (1). One important mechanism is induction of genes that code for proteins with inflammatory, thrombotic and vasoregulatory actions. When endothelial cells are stimulated with bacterial lipopolysaccharide (LPS), or cytokines such as tumor necrosis factor-α (TNF-α), and interleukin 1 (IL-1), transcriptional events mediated by the nuclear factor kappa B (NF-κB) family and other transcription factors are induced (2, 3). Subsequently, the protein products of multiple immediate-early and other genes are synthesized, including adhesion molecules, chemokines, cytokines, and growth factors (2–7). LPS also induces expression of cyclooxygenase-2 (COX-2) (8, 9). Eicosanoids generated from arachidonic acid by COX-2 regulate vascular tone and function, inflammatory events, angiogenesis, and apoptosis (10–12).

In addition to members of the NF-κB family, other transcription factors and enhancers are involved in the regulated expression of gene products by endothelial cells in response to LPS and cytokines (4). This study was conducted to identify, by subtractive hybridization, additional new inducible factors in inflamed endothelial cells.

Retinoic acid-inducible gene I (RIG-I) is a member of DExH box family protein, and was identified as a gene induced by retinoic acid in a promyelocytic leukemia cell line (13). It is designated as a putative helicase from its amino acid sequences. However, details of its biological function are not known. Here we show that RIG-I is induced in human endothelial cells treated with LPS. We also present evidence that RIG-I regulates expression of COX-2.

MATERIALS AND METHODS

Materials. Cell culture medium Humedia EB-2 and its supplements were from Kurabo (Osaka, Japan). LPS from Escherichia coli serotype OIII:B4 was from Sigma (St. Louis, MO). An RNeasy kit, an Effectene transfection reagent and *Taq* DNA polymerase were from Qiagen (Germany). Medium199, fetal bovine serum (FBS), a 5′-RACE kit, M-Mulv reverse transcriptase and primer oligo(dT)$_{12–18}$ were from Life Technologies (Rockville, MD). The plasmid pcDNA3 was from InVitrogen (Carlsbad, CA). Positively charged nylon membrane, digoxigenin (DIG) RNA labeling and detection kits, and a β-gal reporter gene assay kit were from Roche Diagnostics GmbH (Germany). Horseradish peroxidase (HRP)-labeled anti-rabbit IgG and anti-mouse IgG antibodies were from Kirkegaard Perry (Gaith-

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ersburg, MD). A mouse anti-COX-2 monoclonal antibody was from Transduction Laboratories (San Diego, CA). A SuperSignal West Pico chemiluminescence substrate was from Pierce (Rockford, IL). A Northern Max kit was from Ambion (Austin, TX). Oligotex-dT^{30} was from TaKaRa (Shiga, Japan). G418, a reporter plasmid pGL3, and the luciferase kit were from Promega (Madison, WI). The oligonucleotide primers were synthesized by the University of Utah DNA/peptide user facility or Greiner Japan (Kanagawa, Japan).

Cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described (14) with a slight modification (6, 7). An endothelial cell line, HUV-EC-C cells (15), and T24 bladder epithelial cells were generous gifts from the Second Department of Internal Medicine, and the Department of Urology, Hirosaki University School of Medicine, respectively. HUV-EC-C were cultured using Medium199 supplemented with 10% FBS and T24 were cultured using RPMI 1640 with 10% FBS.

Polymerase chain reaction (PCR)-based subtractive cDNA cloning. HUVEC were treated for 4 h with 10 μg/ml LPS or vehicle, and poly(A)$^+$ mRNA was isolated. The selection and amplification of the differentially expressed gene fragments were performed using a PCR-select system (Clontech, Palo Alto, CA) according to the supplier's protocol. First-strand cDNA was synthesized from 2 μg of poly(A)$^+$ mRNA from unstimulated or LPS-stimulated HUVEC, using an *Rsa*I–oligo(dT) primer. After the second strand cDNA was synthesized, the cDNA was digested with *Rsa*I. The *Rsa*I-digested cDNA from LPS-stimulated HUVEC was divided into two parts, and differentially ligated to adaptors provided. The adaptor-ligated cDNAs from LPS-stimulated HUVEC were hybridized with the *Rsa*I-digested cDNA from unstimulated HUVEC. Both of the hybridization reaction mixtures were combined and excess cDNA from unstimulated HUVEC was added. After the second hybridization, the differentially expressed genes were amplified by PCR using primers nested in the two adaptors. The nucleotide sequences of the cDNA fragments were determined. To obtain the full-length cDNA, several rounds of rapid amplification of cDNA ends (RACE) were performed.

Northern blot analysis. Poly(A)$^+$ mRNA was electrophoresed (1 μg/lane) on a 1% agarose gel containing formaldehyde. The RNA was blotted onto a positively charged nylon membrane by capillary transfer, and hybridized with DIG-labeled antisense cRNAs for RIG-I and β-actin using a Northern Max kit. The 405 bp fragment of RIG-I used as a template for DIG-labeled riboprobe synthesis. The synthesis of DIG-labeled probes and the detection were performed according to the specification of the supplier.

Reverse transcriptase-mediated PCR (RT-PCR) analysis. First-strand cDNA was synthesized from 1 μg of total RNA using M-Mulv reverse transcriptase and primer oligo(dT)$_{12-18}$. Primers for COX-2, RIG-I, intercellular adhesion molecule-1 (ICAM-1) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were as follows: COX-2-F (5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′), COX-2-R (5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′), RIG-I-F (5′-GCA TAT TGA CTG GAC GTG GCA-3′), RIG-I-R (5′-CAG TCA TGG CTG CAG TTC TGT C-3′), ICAM-1-F (5′-CAC AGT CAC CTA TGG CAA CG-3′), ICAM-1R (5′-TTC TTG ATC TTC CGC TGG C-3′), GAPDH-F (5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′), and GAPDH-R (5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′).

The reaction conditions were 1× (94°C, 1 min); 30× (94°C, 1 min; 58°C, 1 min; 72°C, 1 min); and 1× (72°C, 10 min). The products were analyzed on a 1.5% agarose gel which was stained with ethidium bromide. The size of the PCR products for COX-2, RIG-I, ICAM-1 and GAPDH was 305, 644, 750, and 598 bp, respectively.

Western analysis. Cells were washed twice with cold PBS and lysed with Laemmli reducing sample buffer. The lysate was subjected to electrophoresis in a 4–20% polyacrylamide gel and the proteins were transferred to a PVDF membrane. Western blotting for RIG-I was performed with rabbit anti-RIG-I antiserum (1:20,000 dilution) and HRP-labeled anti-rabbit IgG antibody. Anti-RIG-I antiserum was prepared from a rabbit immunized with the recombinant GST-RIG-I fusion protein. Western blotting for COX-2 was performed as described (16). Western blotting for actin was performed using an anti-actin monoclonal antibody.

Immunodetection was performed using a SuperSignal West Pico chemiluminescent substrate.

Transfection of RIG-I cDNA into T24 cells. The coding region of the full-length RIG-I cDNA was cloned into mammalian expression vector pcDNA3. RIG-I cDNA was transfected into T24 cells using Effectene and T24 cells retaining the exogenous RIG-I were established after selection with G418 (Geneticin). RT-PCR analysis and Western blot analysis were performed as described above.

Transfection of reporter constructs. pGL3 with no inserts or containing regions from −1838 bp to +129 bp of the human COX-2 promoter (16, 17) were cotransfected with the RIG-I construct into HUV-EC-C cells using Effectene. β-Galactosidase cloned into pHβAPr-1-neo (18) with a human β-actin promoter was used as a control for normalization of transfections. The cells were harvested after 24 h, and assayed for luciferase or β-galactosidase.

RESULTS

RIG-I is induced in endothelial cells stimulated with LPS. Using subtractive hybridization (19), we identified LPS-inducible genes not known to be expressed in this cell type in addition to multiple known LPS-inducible genes such as VCAM-1, ICAM-1, IL-6, IL-8, COX-2. One of these was RIG-I. We first isolated a 402 bp fragment of the cDNA and, subsequently, identified a 2965 bp clone by RACE. This cDNA contained a 2775 bp open reading frame predicted to encode a protein of 925 amino acids with molecular weight of 101 K. Homology search revealed that the cDNA was identical to that of RIG-I (13), a member of DExH-box proteins.

The expression of RIG-I is regulated in endothelial cells stimulated with LPS. RIG-I mRNA was not detectable in unstimulated endothelial cells by Northern analysis, but a transcript of appropriate size was present after 4 h of stimulation with LPS (Fig. 1A). LPS induced the RIG-I transcript and protein in a concentration-dependent manner (Figs. 1B and 1C). RIG-I mRNA was peaked at 4–8 h after the stimulation with LPS, before maximal accumulation of another inducible gene COX-2 (Fig. 2A). The increase of RIG-I protein was lagged somewhat behind mRNA display (Fig. 2B).

RIG-I induces COX-2 expression. DExH-box proteins have regulatory functions in multiple cellular processes involved in new expression of gene products (23, 30). Then, we examined the effect of introducing RIG-I into an readily transfected cell line, T24, that expresses COX-2 in an inducible fashion. RIG-I cDNA was transfected into T24 cells and RIG-I-expressing cells were selected using G418. Overexpression of RIG-I protein in the transfected cells was confirmed by Western analysis (Fig. 3B). Introduction of RIG-I resulted in enhanced expression of COX-2 mRNA and protein (Fig. 3) in this model. In contrast, RIG-I did not

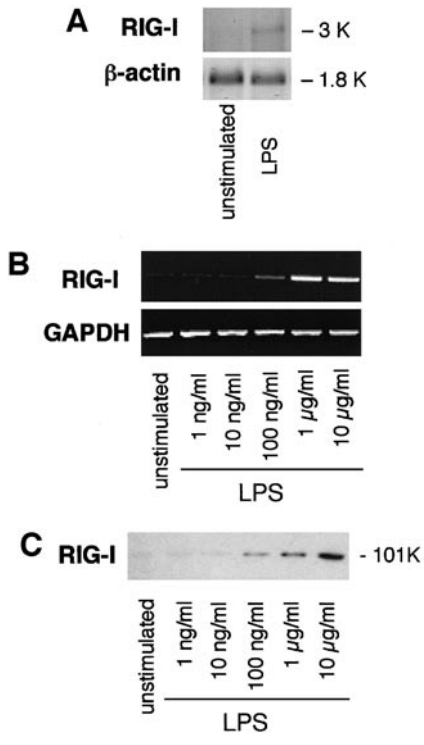

FIG. 1. RIG-I is expressed in human endothelial cells stimulated with LPS in a concentration-dependent manner. (A) Northern blot analysis of mRNAs for RIG-I and β-actin. HUVEC were stimulated with 10 μg/ml LPS for 4 h. Poly(A)$^+$ RNA was isolated and subjected to Northern blot analysis. LPS induced a band for the RIG-I transcript of approximately 3.0 kb. (B) HUVEC were incubated with 1 ng/ml to 10 μg/ml LPS for 4 h, and the cells were subjected to total RNA extraction. Single-strand cDNA was synthesized from 1 μg of total RNA, and RT-PCR analysis of mRNAs for RIG-I and GAPDH was performed. (C) HUVEC were treated with LPS for 16 h. Cells were lysed and subjected to SDS–PAGE, and proteins were transferred to a PVDF membrane. RIG-I protein with a molecular weight of 101 K was detected using rabbit anti-RIG-I antiserum and a HRP-labeled anti-rabbit IgG antibody.

alter expression of the mRNA for ICAM-1, an inducible gene that is basally expressed in T24 cells (Fig. 3A).

RIG-I triggers COX-2 promoter activity in endothelial cells. We examined the ability of RIG-I to regulate COX-2 expression using reporter constructs consisting of the COX-2 promoter in HUV-EC-C. Cotransfection with RIG-I cDNA resulted in about 3.9-fold increase in the COX-2 promoter activity compared to the activity triggered by the control plasmid (Fig. 4).

DISCUSSION

LPS induces multiple genes in human endothelial cells and other cell types (20). The products of these induced genes mediate vascular and inflammatory responses in bacteremia and the sepsis syndrome (21). We identified RIG-I as a product of LPS-stimulated endothelial cells. The RIG-I cDNA was previously cloned as a gene induced by retinoic acid during the differentiation of an acute promyelocytic leukemia cell line (13). RIG-I has DExH motif and is a member of the DExH-box family of proteins (22–25). RIG-I also has GxGKT motif. The DExH and GxGKT sequences confer binding and hydrolysis of nucleoside triphosphates (24, 26). Preliminary experiments demonstrate that RIG-I binds ATP (data not shown). In conjunction with other sequences, the conserved motifs may also confer nucleic acid helicase activity to DExH-box gene products (27–31). While some family members have been shown to unwind nucleic acid segments in *in vitro* experiments (25, 31), in other cases a helicase function has been sought for but not found. The DExH-box factors and related proteins have also been implicated in transcriptional activation and repression, chromatin remodeling, release of transcripts from elongation complexes, conformational changes in spliceosomes or disruption of RNA–protein interactions, and in RNA processing, transport, translation and decay (24, 25, 30, 31). Dysregulation of DExH-box proteins may contribute to pathologic disorders that include xeroderma pigmentosum and Bloom's syndrome (28, 32).

Porcine homolog of RIG-I was identified as a putative porcine RNA helicase induced by virus (RHIV-1), which was induced in alveolar macrophages by infection with porcine reproductive and respiratory syndrome virus (33). The expression and functions of RIG-I have not been characterized in vascular endothelial cells.

In the present study, we found that LPS induced the expression of RIG-I mRNA and protein in HUVEC. This result suggests that RIG-I has important functional roles in activated and inflamed endothelial cells. Next we examined the possibility that RIG-I may influence expression of other genes that are induced in a regulated fashion in endothelium, such as COX-2.

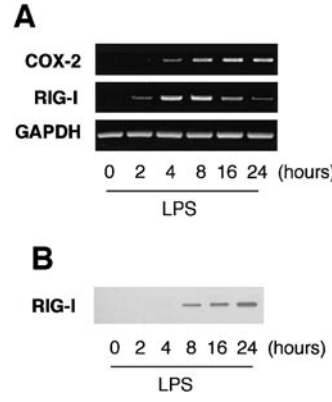

FIG. 2. RIG-I is expressed in HUVEC stimulated with LPS in a time-dependent fashion. (A) HUVEC were stimulated with 10 μg/ml LPS for the times shown, and RT-PCR analysis for RIG-I, COX-2 and GAPDH was performed. (B) Cell lysate was prepared and subjected to Western blot analysis for RIG-I.

Overexpression of RIG-I in T24 cells resulted in enhanced expression of COX-2 mRNA and protein whereas a second inducible gene, ICAM-1, was not altered. In an endothelial cell line, transfection of RIG-I stimulated COX-2 promoter activity. These results suggest that RIG-I may play a role in the expression of COX-2, at least in part, at the transcriptional level in endothelial cells.

COX-2 expression is regulated by NF-κB, NF-IL6, CRE, SP1, HMG-I(Y), PPARα and additional factors (34–39), and also by posttranscriptional mechanisms (40–42). Upregulation of the COX-2 by RIG-I indicates additional levels of complexity in control of this gene. The critical actions of the COX-2 enzymatic activity in inflammation, development, angiogenesis, and cell regulation are consistent with multiple control mechanisms and points of regulation (11, 12, 39). The mechanisms by which RIG-I enhances COX-2 expression remain to be determined.

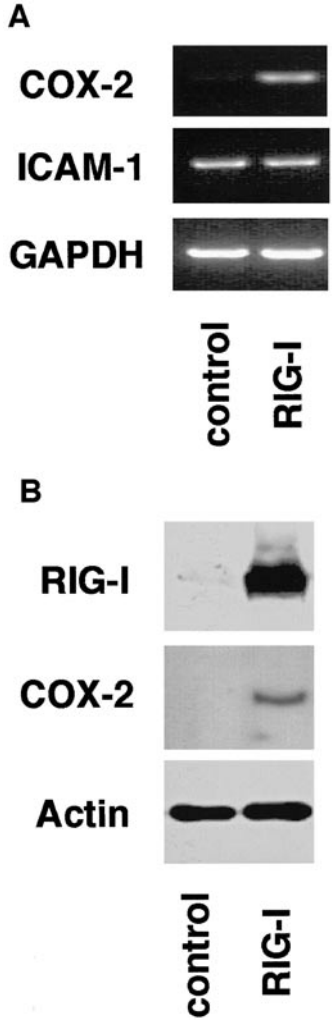

FIG. 3. RIG-I enhances expression of COX-2. RIG-I cDNA was transfected into T24 cells and colonies were established after selection using G418. (A) Total RNA was extracted and RT-PCR analysis for COX-2 and ICAM-1 was performed. (B) Cell lysate was prepared and subjected to Western blot analysis for RIG-I, COX-2 and actin.

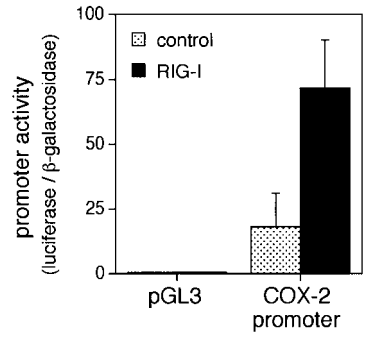

FIG. 4. RIG-I enhances COX-2 promoter activity in endothelial cells. Regions from −1838 bp to +129 bp of the human COX-2 promoter were ligated into a pGL3 vector. HUV-EC-C cells were transfected with the RIG-I expression vector or a control plasmid together with the COX-2 reporter construct or the empty pGL3. Cells were cotransfected with a β-galactosidase expression construct to standardize for transfection efficiency. After 24 h, the cells were lysed and the activities for luciferase and β-galactosidase were assayed. The results are expressed as the ratio of luciferase to β-galactosidase, and means (±SD) of three different experiments are shown. When the cells were transfected with a control pGL3, without insertion, luciferase activity was virtually negative regardless of the RIG-I cotransfection. Cotransfection of RIG-I cDNA enhanced the COX-2 promoter activity (about 3.9-fold) compared to the control plasmid.

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