RORα1 and RORα4 suppress TNF-α-induced VCAM-1 and ICAM-1 expression in human endothelial cells

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Abstract Retinoic acid receptor-related orphan receptor-α (RORα) is a nuclear orphan receptor. Adenovirus-mediated overexpression of RORα1 and RORα4 suppressed tumor necrosis factor-α (TNF-α)-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in human umbilical vein endothelial cells. Overexpression of RORα1 and RORα4 also suppressed TNF-α-stimulated translocation of p50 and p65 to the nucleus. In contrast, dominant-negative deletion mutants of RORα1 and RORα4 failed to suppress the induction of VCAM-1 and ICAM-1 and translocations of p50 and p65. These results suggest that RORα1 and RORα4 regulate the inflammatory responses via inhibition of the nuclear factor-κB signaling pathway in endothelial cells.

Key words: Nuclear receptor; Endothelial cell; Vascular cell adhesion molecule-1; Intracellular adhesion molecule-1; Nuclear factor-κB; Activator protein-1

1. Introduction

Endothelial cells (ECs) are stimulated by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-6 to express adhesion molecules for leukocytes and other inflammatory cells such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) [1,2]. Increased expression of adhesion molecules on ECs leads to leukocyte recruitment via interactions with their cognate ligands on leukocytes at the sites of atherosclerosis [3,4]. The VCAM-1 and ICAM-1 promoters contain binding sites for two major transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [5–8]. NF-κB is an inducible dimeric transcription factor of the Rel/NF-κB family [9], and activated NF-κB is present in atherosclerotic lesions [10]. It is involved in the regulation of several pro-inflammatory genes in ECs [11]. AP-1 proteins are dimers of Jun and Fos family members [12] and are regulated by growth factors, cytokines, and oxidative stress [13]. Thus, NF-κB and AP-1 both play roles in regulating the inflammatory response in vasculature.

Recently, activators of nuclear receptors, such as the estrogen receptor, glucocorticoid receptor, retinoid receptor, and peroxisome proliferator-activated receptor (PPAR) [14–18], have been shown to inhibit the expression of several adhesion molecules in vascular cells, possibly mediated by inhibiting the NF-κB signaling pathway and/or AP-1 signaling pathway. Therefore, it has been suggested that nuclear receptors have a potential role in modulating pro-inflammatory gene expression at inflammatory sites in the vascular wall during atherosclerosis and other inflammatory diseases.

Retinoic acid receptor-related orphan receptor (ROR) belongs to the steroid hormone nuclear receptor superfamily [19]. There are three ROR genes, RORα, RORβ, and RORγ. The human RORα gene encodes at least four distinct splicing isoforms, RORα1, RORα2, RORα3, and RORα4 (also termed RZRα) [20–22]. These isoforms share common DNA-binding domains (DBDs) and putative ligand-binding domains (LBDs), but differ in their N-terminal domains. RORα usually binds as a monomer to a ROR response element (RORE) composed of a 6 bp A/T-rich sequence immediately preceding a site with the core motif of RGGTCA (R = A or G) [21]. The four isoforms of RORα display different binding preferences and transcriptional activities. *Staggerer* mice, whose RORα gene has a deletion in the RORα LBD sequence causing a frame shift in the protein, are characterized by severe cerebella ataxia due to a developmental defect in Purkinje cells [23]. The mice have an increased susceptibility to atherosclerosis [24] and show alterations in several immune responses [25]. Recently it was reported that RORα suppressed TNF-α-induced expression of COX-2, IL-6, and IL-8 in vascular smooth muscle cells (VSMCs) in part by upregulating IκBα gene expression [26]. Furthermore RORα expression in atherosclerotic plaque was found to be significantly decreased [27]. Therefore, RORα may play an important role in modulating inflammatory responses in vasculature. There is little information, however, about the effects of RORα on vascular ECs. In this study, we show that RORα1 and RORα4 are present in human umbilical vein endothelial cells (HUVECs) and that adenovirus-mediated overexpression of RORα1 and RORα4 suppresses TNF-α-induced expression of VCAM-1 and ICAM-1 via inhibiting NF-κB activation thus regulating the inflammatory response.

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Abbreviations: AP-1, activator protein-1; DBD, DNA-binding domain; HUVECs, human umbilical vein endothelial cells; ICAM-1, intracellular adhesion molecule-1; LBD, ligand-binding domain; NF-κB, nuclear factor-κB; RORα, retinoic acid receptor-related orphan receptor-α; RORE, ROR response element; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VSMCs, vascular smooth muscle cells

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2. Materials and methods

2.1. Cells and materials

HUVECs (Clonetics, San Diego, CA, USA) were maintained in EBM-2 Bullet Kit (Clonetics). CV-1 cells (ATCC CCL-70) were cultured with Eagle's minimum essential medium containing 10% fetal bovine serum and non-essential amino acids. TNF-α was purchased from R&D Systems (Abingdon, UK).

2.2. Adenovius generation and infection

RORα1 and RORα4 cDNA were cloned from human fetal liver (Human Quick-Clone cDNA, Clontech, Palo Alto, CA, USA) and inserted into the vector, pcDNA (Invitrogen, Carlsbad, CA, USA). RORα1Δ and RORα4Δ were constructed by deleting the fragment between the *Nco*I site and the C-terminal end and inserted into the pcDNA vector. Recombinant adenoviruses (Ad-RORα1, Ad-RORα1Δ, Ad-RORα4, and Ad-RORα4Δ) were constructed with the Adeno-X Expression System (Clontech) according to the manufacturer's instructions. Ad-lacZ (Clontech) was used as the control in each experiment. HUVECs were infected with these adenoviruses at a multiplicity of infection of 100.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from HUVECs with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR analysis was performed with 15 ng total RNA and the GeneAmpEZ rTth RNA PCR Kit (Applied Biosystems, Foster, CA, USA). The specific primer sets for the target genes were as follows: for RORα1, 5′-ACCCCGCTGAACCAGGAATC-3′ and 5′-GAAGTTCCGTCAGCCCGTTG-3′ (505 bp products); for RORα2, 5′-CCTGGAGGCAGAATGGCAAG-3′ and 5′-CCGTTGGCCGAGATGTTGTA-3′ (451 bp); for RORα3, 5′-AACTGGGATGGAGCCACAGC-3′ and 5′-CCGTTGGCCGAGATGTTGTA-3′ (445 bp); for RORα4, 5′-CTCCGCACCGCGCTTAAT-3′ and 5′-GAAGTTCCGTCAGCCCGTTG-3′ (441 bp); for RORα-LBD, 5′-CAGAGTGTGCCGTGCCTTTG-3′ and 5′-TCAATTTGCATTGCTGGCTCA-3 (468 bp). RT-PCR was carried out by incubating the reaction mixture for 30 min at 60°C and 60 s at 94°C, followed by 35 cycles of 15 s at 94°C and 15 s at 60°C. The RT-PCR products were analyzed by electrophoresis in a 2.5% agarose gel followed by ethidium bromide staining.

Quantitative real-time RT-PCR was performed with 15 ng total RNA, the QuantiTect SYBR Green RT-PCR Kit (Qiagen), and the specific primer sets for the target genes were as follows: for VCAM-1, 5′-TGGGCTGTGAATCCCCATCT-3′ and 5′-GGGTCAGCGCGTGGAATTGGTC-3′; for ICAM-1, 5′-CGTGGGGAGAAGGAGCTGAA-3′ and 5′-CAGTGCGGCACGAGAAATTG-3; for IκBα, 5′-CCTGCAGCAGACTCCACTCC-3′ and 5′-CAGGCAGCCCTGCTCACAG-3′. RT-PCR was carried out by incubating the reaction mixture for 30 min at 60°C and 15 min at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 58°C, and 30 s at 72°C. GAPDH mRNA levels were measured with TaqMan EZ RT-PCR Kit (Applied Biosystems) and Pre-Developed TaqMan Assay Reagents Control Kit (Applied Biosystems). The mRNA expression levels were normalized by GAPDH expression and presented as the relative expression level compared to the expression levels obtained from the Ad-lacZ control.

2.4. Transcriptional assay

CV-1 cells were transfected using 4 μl of FuGENE6 (Roche, Basel, Switzerland), with 1 μg pTK-RORE-Luc plasmid, which contained a thymidine kinase promoter controlling the expression of luciferase with RORE (4 tandem copies of TCGCAAAATGGGTCACGG), mixed with 0.1–0.3 μg of pcDNA encoding RORα1, RORα1Δ, RORα4, or RORα4Δ. The transfected cells were cultured in Eagle's minimum essential medium containing 2% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT, USA) and non-essential amino acids. Luciferase activity was measured after incubation for 48 h (Dual Luciferase Assay Kit, Promega, Madison, WI, USA). In the experiments, 0.05 μg pRL-TK (*Renilla* luciferase reporter, Promega) was included in the transfection mixture, and the promoter-dependent transcriptional activity was normalized to *Renilla* luciferase activity.

2.5. Western blot analysis

Cytoplasmic and nuclear extracts were prepared from HUVECs using CellLyticNu-CLEAR Extraction Kit (Sigma, St. Louis, MO, USA). The extracts (cytoplasmic protein, 2.5 μg; nuclear protein, 10 μg) were analyzed by Western blot analysis using the specific antibody against RORα (sc-6062), VCAM-1 (sc-13160), ICAM-1 (sc-8439), NF-κB p50 (sc-8414), NF-κB p65 (sc8008), IκBα (sc-1643), Jun (sc-45), or phosphorylated Jun (sc-7981) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and detected by chemiluminescence.

2.6. Statistical analysis

Quantitative data were expressed as means ± S.D. from three or four experiments. Statistical analysis was performed using the ANOVA program, and probability values were calculated using the Dunnet test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Specific RORα isoforms are expressed in HUVECs

We first investigated whether RORα is expressed in human ECs and if so, which of the four RORα isoforms was present. Using specific primers for RORα isoforms, we determined that RORα1 and RORα4 were expressed in HUVECs by RT-PCR (Fig. 1A). In contrast, expression of both RORα2 and RORα3 was undetectable, even if the input of RNA or number of PCR cycles was increased. These results show that RORα1 and RORα4 were the main transcripts of the RORα gene in HUVECs.

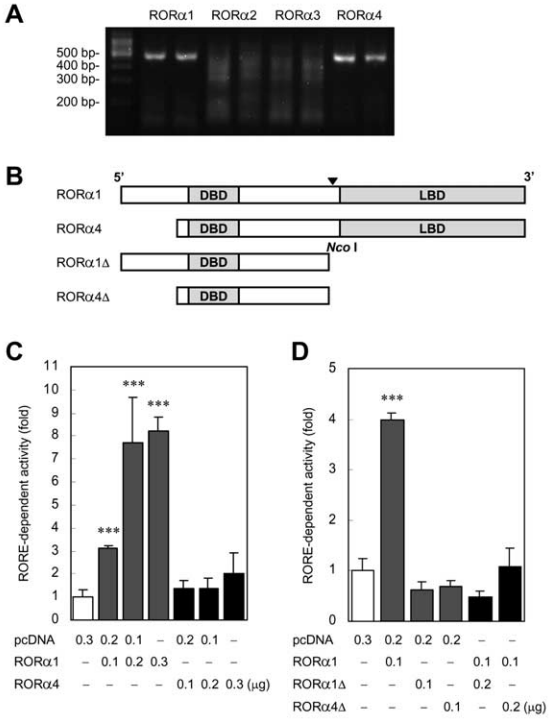

Fig. 1. A: RORα1 and RORα4 are expressed in HUVECs. Total RNA was analyzed by RT-PCR as described. B: Schematic representation of RORα1, RORα4, and their deletion mutants, RORα1Δ and RORα4Δ. The black triangle indicates the start of the deletion of RORα transcripts from *Staggerer* mice. C,D: RORα1 and RORα4 activate the RORE-dependent transcription. CV-1 cells were co-transfected with the pTK-RORE-Luc plasmid (1 μg), pRL-TK (0.05 μg) and pcDNA and/or pcDNA encoding RORα1, RORα4, RORα1Δ, or RORα4Δ as indicated. The results are presented as fold induction of the basal activity of pcDNA. ***$P < 0.001$ versus pcDNA.

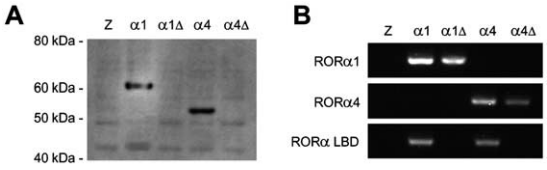

Fig. 2. Adenovius-mediated gene expression of RORα1 and RORα4 in HUVECs. HUVECs were infected with the recombinant adenoviruses and cultured for 24 h. Western blotting analysis was performed on the nuclear extracts (A) and RT-PCR analysis was preformed on the total RNA (B) as described. Z, Ad-lacZ; α1, Ad-RORα1; α1Δ, Ad-RORα1Δ; α4, Ad-RORα4; α4Δ, Ad-RORα4Δ.

3.2. *The transactivation activities of RORα1 and RORα4 are mediated by their LBDs*

To test the functionality of cloned RORα1 and RORα4, we analyzed the ability of RORα1 and RORα4 to transcribe a luciferase reporter plasmid, in which the promoter contains RORE (4×) whose specific sequence was derived from the promoter of 5-lipoxygenase [28]. Co-transfection in CV-1 cells of the reporter plasmid and the expression vector containing RORα1 led to RORE-dependent luciferase activity (Fig. 1C). In contrast, when the RORα4 expression vector was co-transfected with the luciferase reporter plasmid, there was an increase of only two-fold at the highest plasmid concentration in luciferase activity. These data are consistent with previous results [21,22], in which it was shown that RORα1 promoted RORE-dependent transcription but the effect of RORα4 was much weaker. To demonstrate that this was specific transcription due to RORα, we next constructed two expression plasmids encoding mutant RORα, RORα1Δ and RORα4Δ, in which their LBDs were deleted (Fig. 1B). The mutations are functionally equivalent to the deletion mutation that occurs in the *Staggerer* mouse, in which RORα has been shown to be inactive as a transcription factor [23]. Co-transfection of either RORα1Δ or RORα4Δ with a luciferase reporter plasmid shows that these mutants have lost their transcriptional activity and cannot transcribe luciferase (Fig. 1D). Our results indicate that both RORα1Δ and RORα4Δ require the LBD for their transactivation activity.

3.3. *The dominant-negative effect of RORα1Δ or RORα4Δ*

Furthermore, we found that co-transfection of RORα1 with either RORα1Δ or RORα4Δ and the luciferase reporter plasmid into CV-1 cells attenuated the RORα1-dependent transcriptional activity (Fig. 1D). These results indicated that RORα1Δ and RORα4Δ acted as dominant-negative forms able to inhibit the activity of RORα1. Thus RORα1Δ and RORα4Δ are excellent tools to investigate RORα function in human ECs.

3.4. *RORα1 and RORα4 suppress VCAM-1 and ICAM-1 expression induced by TNF-α in HUVECs*

Since RORα1 and RORα4 were the major isoforms of RORα present in HUVECs, we next examined whether RORα1 and RORα4 could regulate pro-inflammatory genes. Because of the difficulties in transfecting primary ECs with plasmids, we developed recombinant adenoviruses that encoded RORα1 and RORα4 as well as their deletion mutants, RORα1Δ and RORα4Δ. At 24 h after infection with Ad-lacZ, almost all of the HUVECs were expressing β-gal (data not shown). Expression of RORα1 and RORα4 protein in HUVECs was detected by Western blotting analysis with an antibody directed against the C-terminal region of RORα allowing detection of all isoforms of RORα but not RORα1Δ and RORα4Δ (Fig. 2A). However, RT-PCR analysis indicated that they were expressed in HUVECs (Fig. 2B).

We then analyzed the expression of VCAM-1 and ICAM-1 by quantitative real-time RT-PCR. VCAM-1 and ICAM-1 expression were highly induced by 1 ng/ml TNF-α treatment for 6 h (VCAM-1: 1100-fold, ICAM-1: 170-fold; Fig. 3A,B). We found that TNF-α-induced VCAM-1 expression was completely suppressed by infection with Ad-RORα1 (99.8% reduction compared to TNF-α-stimulated Ad-lacZ; Fig. 3A). VCAM-1 induction was reduced in Ad-RORα4-infected HUVECs (73% reduction). In contrast, Ad-RORα1Δ and Ad-RORα4Δ failed to suppress the induction of VCAM-1. As shown in Fig. 3B, Ad-RORα1 also suppressed TNF-α-induced ICAM-1 expression in HUVECs (74% reduction), but Ad-RORα4 was slightly less effective in reducing ICAM-1 induction (49% reduction). The levels of VCAM-1 and ICAM-1 protein expression were confirmed by Western blotting analysis (Fig. 3C). These data indicate that the presence of RORα1 or RORα4 can prevent the TNF-α-induced expression of VCAM-1 and ICAM-1 in HUVECs, but that their dominant-negative mutants could not suppress the induction of VCAM-1 and ICAM-1.

3.5. *The suppression of VCAM-1 and ICAM-1 expression by RORα is mediated by inhibiting nuclear translocation of p50/p65*

To determine the mechanisms by which RORα1 and RORα4 inhibited TNF-α-induced VCAM-1 and ICAM-1 expression, we examined whether RORα could inhibit NF-κB activation. NF-κB is composed of the p50 and p65 subunits and translocation of the complex to the nucleus is one of the critical steps in activating the NF-κB signaling pathway. As

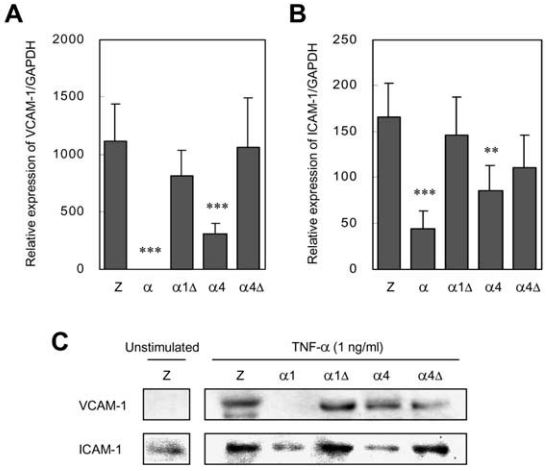

Fig. 3. RORα1 and RORα4 inhibit TNF-α-induced expression of VCAM-1 and ICAM-1 in HUVECs. HUVECs were infected with the recombinant adenoviruses and cultured for 18 h. Total RNA was prepared after treatment with 1 ng/ml TNF-α for 6 h, and analyzed by quantitative real-time RT-PCR as described. The normalized expression of VCAM-1 (A) and ICAM-1 (B) by GAPDH is presented as the relative expression to their basal expression in Ad-lacZ-infected and unstimulated cells. $^{**}P < 0.01$, $^{***}P < 0.001$ versus Ad-lacZ. C: The cytoplasmic extracts of the HUVECs were prepared and then VCAM-1 and ICAM-1 were detected with specific antibodies.

expected, TNF-α treatment increased the nuclear abundance of p50 and p65 in HUVECs (Fig. 4) and this translocation was clearly suppressed by overexpression of RORα1 or RORα4. In contrast, Ad-RORα1Δ and Ad-RORα4Δ failed to suppress the translocation of p50 and p65 to the nucleus. The retention of p50 and p65 in the cytoplasm was not changed whichever form of RORα was overexpressed. Therefore, these data indicate that RORα1 and RORα4, but not their dominant-negative deletion mutants, inhibited NF-κB signal activation without regulating p50 and p65 protein expression.

3.6. *Inhibition of p50/p65 nuclear translocation by RORα overexpression is not mediated by upregulating IκBα expression*

It has been suggested that the anti-inflammatory effect of RORα is mediated by upregulation of IκBα expression in VSMCs [26]. To test this, we then determined whether RORα1 and RORα4 could upregulate IκBα expression in ECs. In unstimulated cells, IκBα complexes with NF-κB and inhibits the translocation of NF-κB to the nucleus. We analyzed the expression levels of IκBα by quantitative real-time RT-PCR and Western blot analysis. Fig. 5 shows that IκBα mRNA expression in HUVECs was upregulated 11-fold by TNF-α treatment for 6 h. Surprisingly, RORα1 and RORα4 clearly repressed both TNF-α-induced IκBα mRNA (RORα1, 78% reduction; RORα4, 79% reduction) and IκBα protein. In contrast, overexpression of either RORα1Δ or RORαΔ did not reduce the expression of IκBα mRNA and protein induced by TNF-α. Therefore, in contrast to the previous observations in VSMCs, these results indicated that RORα1 and RORα4 negatively regulated TNF-α-induced IκBα expression.

3.7. *RORα1 reduces phosphorylation and translocation of c-Jun*

Since the expression of VCAM-1 and ICAM-1 are regulated not only by NF-κB but also by another transcriptional factor, AP-1 [6,8], we tested the effect of RORα overexpression on AP-1 activation. AP-1 protein is a dimer of the Jun and Fos families, and its transcription activity is correlated with the translocation and phosphorylation of c-Jun at Ser 63 and Ser 73 by the Jun-N-terminal kinase [29]. Overexpression of RORα1, but not RORα4, in HUVECs by adenovirus vectors decreased c-Jun protein contents in the nucleus (Fig. 4).

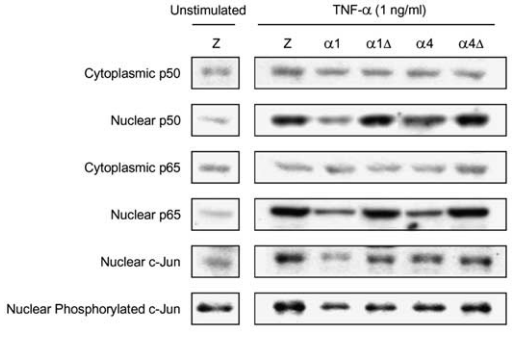

Fig. 4. RORα1 and RORα4 suppress TNF-α-induced translocation of p50, p65, and c-Jun in HUVECs. HUVECs were treated as described in Fig. 3, and then cytoplasmic and nuclear extracts were prepared. p50, p65, c-Jun, and phosphorylated c-Jun were detected with specific antibodies.

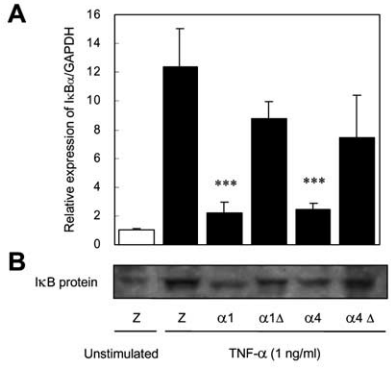

Fig. 5. RORα1 and RORα4 inhibit TNF-α-induced IκBα expression in HUVECs. HUVECs were treated as described in Fig. 3. Quantitative real-time RT-PCR analysis (A) and Western blot analysis (B) were performed. ***$P < 0.001$ versus Ad-lacZ-infected and TNF-α-treated cells.

Furthermore, the overexpression reduced c-Jun phosphorylation at Ser 73 (Fig. 4). As expected, RORα1Δ and RORα4Δ had no effect on the nuclear abundance and the phosphorylation of c-Jun. Thus, these data suggest that RORα1 suppressed not only the NF-κB signaling pathway but also the AP-1 signaling pathway.

4. Discussion

Several studies have indicated that nuclear receptors including estrogen receptor, glucocorticoid receptor, retinoid receptor, and PPAR, inhibit the expression of genes involved in the inflammatory response in vascular cells [14–18]. In contrast, the effect of RORα on expression of these genes in vascular cells has been poorly studied, and, in particular, the role of RORα on expression of adhesion molecules has not been described. RORα1 can activate the expression of genes, which have a RORE in their promoter region, and while RORα4 can also bind the same sequences its activity is much weaker than that of RORα1. Recently, Besnard et al. [27] found that human aortic VSMCs and ECs expressed RORα1 and RORα4 but did not express RORα2 and RORα3. In this report, we have confirmed the presence of RORα1 and RORα4 in HUVECs, which suggests RORα, and, in particular, RORα1 might play a role in modulating gene expression in vascular cells.

The adhesion molecules VCAM-1 and ICAM-1 are highly regulated at the transcriptional level by a number of mediators, such as TNF-α [6,7]. Recent promoter studies have characterized the 5′ regulatory region of VCAM-1 and ICAM-1. TNF-α induces binding of NF-κB to the promoter region of VCAM-1 and ICAM-1 [5,7]. By adenovirus-mediated gene transfer of RORα1 and RORα4, we showed that overexpression of RORα1 and RORα4 downregulated TNF-α-induced VCAM-1 and ICAM-1 expression in HUVECs. In contrast, overexpression of their deletion mutants of LBD did not downregulate the induction of VCAM-1 and ICAM-1. The inhibitory effects of these RORα proteins correlate with their transcriptional activities determined on a luciferase reporter gene (Fig. 1C). This is the first report demonstrating that RORα regulates the expression of adhesion molecules in ECs and that the LBD is necessary for their regulation.

We then investigated the role of the NF-κB system in con-

trolling the TNF-α induction of the adhesion molecules and showed that overexpression of RORα1 and RORα4 negatively regulated the TNF-α-stimulated translocation of p50 and p65 into the nucleus. Recently, Delerive et al. [26] reported that RORα1 suppressed TNF-α-induced IL-6, IL-8, and COX-2 expression in VSMCs. They suggested that the mechanism of action was mediated via the transcriptional upregulation of IκBα by RORα1. In contrast to their findings, we demonstrated that RORα1 downregulated TNF-α-induced IκBα expression. In addition, we observed that overexpression of RORα1 and RORα4 did not upregulate the basal expression of IκBα but we confirmed that RORα1 and RORα4 overexpression suppressed TNF-α-induced expression of other NF-κB-regulated genes such as IL-6 and COX-2, in HUVECs (data not shown). The suppression of these inflammatory response genes is clearly due to inhibition of p50 and p65 nuclear translocation. Therefore, our data confirm Delerive et al.'s data [26] that RORα1 negatively regulates the inflammatory response. However, the regulation in ECs treated with TNF-α was not due to transcriptional upregulation of IκBα expression. It is known that IκBα is not only an inhibitor of NF-κB but also its expression is modulated by NF-κB [9]. The promoter region of IκBα has a NF-κB binding site. TNF-α treatment increases IκBα expression via NF-κB activation [30]. Our data confirm that TNF-α treatment upregulates IκBα but that RORα1 and RORα4 overexpression does not upregulate IκBα. Furthermore, RORα1 and RORα4 suppress TNF-α-induced IκBα upregulation in ECs. It is possible that NF-κB activation by TNF-α was quickly suppressed by RORα and in consequence IκBα upregulation was prevented. Alternatively, other negative regulatory element may exist in the IkBα promoter region. Our results clearly show that vascular ECs RORα1 and RORα4 inhibit NF-κB-induced gene expression and that the inhibition is not due to upregulation of IκBα expression levels. Rather, the inhibition of NF-κB activation may result from inhibition of IκBα phosphorylation and/or degradation.

AP-1 is a family of transcriptional factors composed of various dimers of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) [14]. These family members bind to DNA containing a TPA (12-*O*-tetradecanoyl-phorbol-13-acetate)-responsive element to regulate gene expression. AP-1 responds to a variety of pathological stimuli, and TNF-α stimuli lead to AP-1 activation and induce VCAM-1 and ICAM-1 expression [6,8]. We demonstrated that RORα1 suppressed the translocation and the phosphorylation of c-Jun in HUVECs. This report shows that RORα1 inhibits AP-1 activation, suggesting that it may have the potential to suppress the effects of many pathological stimuli.

In this report, we describe mechanisms by which the NF-κB signaling pathway is inhibited by RORα1 and RORα4. RORα could regulate the signaling pathway in two ways. First, through their transcription activity, RORα1 and RORα4 may regulate an inhibitory molecule(s) of NF-κB. RORα1 stimulates RORE-dependent transcriptional activity and RORα4 weakly activates transactivation. In addition, the LBD-deletion mutants of RORα1 and RORα4 failed to stimulate transcriptional activity, to suppress gene expression, and to translocate p50 and p65. Thus their LBDs are essential for both their transactivation and translocation activity. The regulation of the NF-κB signaling pathway by RORα1 and RORα4 may correspond to the levels of their transcription activity (RORα1 > RORα4 ≫ RORαΔ). Thus it is plausible that RORα regulates an inhibitory molecule(s) of the NF-κB signaling pathway. The inhibitor could be a molecule that blocks the activity of either RIP, TRAF1 or 2, TAK1, IKK, or blocks IκB degradation through upregulation of IκB expression, inhibition of IκB phosphorylation, inhibition of IκB ubiquitination or inhibition of the proteosome. We report in this manuscript that IκBα expression is not upregulated by RORα. We also found that RORα did not change the expression level of IKKβ (data not shown). It would be of interest to explore further which molecule is responsible for inhibiting NF-κB activity mediated by RORα. Second, RORα may regulate NF-κB signaling pathway through the LBD of RORα independently of its transactivation activity by a mechanism such as direct interaction of p50/p65 with RORα. We have shown that the LBD is essential for p50/p65 nuclear translocation (Fig. 4). Analogously, PPARα physically interacts with p65 and c-Jun [31] and PPARγ also bound p50 and p65 [32]. These reports suggest that the interference with NF-κB transactivation by PPAR might involve the direct binding to p50/p65. Nuclear receptors including retinoic acid receptor and Rev-erb, which are closely homologous to RORα, have been reported to have a nuclear location signal within DBD [33]. It is not known whether RORα also has a nuclear location signal within the DBD. Regardless, we cannot exclude the possibility that the LBD deletion mutant may be retained in the cytoplasm. In that case, the ROR mutant may lose transactivation activity due to a defect in nuclear translocation. We have shown loss of function of the deletion mutant (Fig. 1C). However, the deletion mutant that was retained in the cytoplasm would not antagonize wild-type RORα1. We have shown a dominant-negative effect in Fig. 1D, suggesting that the deletion mutant was expressed and translocated into the nucleus where it competed with the wild-type RORα1 for RORE binding. Thus, we believe that there is strong circumstantial evidence that RORα1Δ and RORα4Δ are translocated to the nucleus. Further insight into the inhibition mechanisms of the interaction of RORα with p50/p65 warrants investigation.

In conclusion, our study demonstrates that overexpression of RORα1 and RORα4 inhibits TNF-α-induced expression of VCAM-1 and ICAM-1 in HUVECs by inhibiting the NF-κB signaling pathway. These results provide insights into the role that RORα plays in regulation of inflammatory response in vascular ECs.

References

[1] Carlos, T.M. and Harlan, J.M. (1994) Blood 84, 2068–2101.
[2] Springer, T.M. (1994) Cell 76, 301–314.
[3] Van der Wal, A.C., Das, P.K., Tigges, A.J. and Becker, A.E. (1992) Am. J. Pathol. 141, 1427–1433.
[4] Collins, T., Read, M.A., Neish, A.S., Whitley, M.Z., Thanos, D. and Maniatis, T. (1995) FASEB J. 9, 889–909.
[5] Neish, A.S., Read, M.A., Thanos, D., Pine, R., Maniatis, T. and Collins, T. (1995) Mol. Cell. Biol. 15, 2558–2569.
[6] Ahmad, M., Theofanidis, P. and Madford, R.M. (1998) J. Biol. Chem. 273, 4616–4621.
[7] Ledebur, H. and Parks, T. (1995) J. Biol. Chem. 270, 933–943.
[8] Wang, N., Verna, L., Hardy, S., Forsayeth, J., Zhu, Y. and Stemerman, M.B. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2078–2084.
[9] Perkins, N.D. (2000) Trends Biochem. Sci. 25, 434–440.
[10] Brand, K., Page, S., Rogler, G., Bartsch, A., Brandl, R., Knue-

chel, R., Page, M., Kaltschmidt, C., Baeuerle, P.A. and Neumeier, D. (1996) J. Clin. Invest. 97, 1715–1722.
[11] Collins, T. (1993) Lab. Invest. 68, 499–508.
[12] Shaulian, E. and Karin, M. (2002) Nat. Cell Biol. 4, E131–E136.
[13] Lo, Y.Y.C. and Cruz, T.F. (1995) J. Biol. Cham. 270, 11727–11730.
[14] Caulin-Glaser, T., Watson, C.A., Pardi, R. and Bender, J.R. (1996) J. Clin. Invest. 98, 36–42.
[15] Cronsten, B.N., Kimmel, S.C., Levin, R.I., Martiniuk, F. and Weissmann, G. (1992) Proc. Natl. Acad. Sci. USA 89, 9991–9995.
[16] Xu, X., Otsuki, M., Saito, H., Sumitani, S., Yamamoto, H., Asanuma, N., Kouhara, H. and Kasayama, S. (2001) Endoclonology 142, 3332–3339.
[17] Gille, J., Paxton, L.L.L., Lawley, T.J., Caughman, S.W. and Swerlick, R.A. (1997) J. Clin. Invest. 99, 492–500.
[18] Marx, N., Sukhova, G.K., Collins, T., Libby, P. and Plutzky, J. (1999) Circulation 99, 3125–3131.
[19] Jetten, A.M., Kurebayashi, S. and Ueda, E. (2001) Prog. Nucleic Acid Res. Mol. Biol. 69, 205–247.
[20] Beckerandre, M., Andre, E. and Delamarter, J.F. (1993) Biochem. Biophys. Res. Commun. 194, 1371–1379.
[21] Giguere, V., Tini, M., Flock, G., Ong, E., Evans, R.M. and Otulakowski, G. (1994) Genes Dev. 8, 538–553.
[22] Carlberg, C., Hooft van Huijsduijnen, R., Staple, J.K., DeLamarter, J.F. and Becker-Andre, M. (1994) Mol. Endocrinol. 8, 757–770.
[23] Hamilton, B.A., Frankel, W.N., Kerrebrock, A.W., Hawkins, T.L., Fitzhugh, W., Kusumi, K., Russell, L.B., Mueller, K.L., van Berkel, V., Birren, B.W., Kruglyak, L. and Lander, E.S. (1996) Nature 379, 736–739.
[24] Mamontova, A., Seguret-Mace, S., Esposito, B., Chaniale, C., Bouly, M., Delhaye-Bouchaud, N., Luc, G., Staels, B., Duverger, N., Mariani, J. and Tedgui, A. (1998) Circulation 98, 2738–2743.
[25] Trenkner, E. and Hoffmann, M.K. (1986) J. Neurosci. 6, 1733–1737.
[26] Delerive, P., Monte, D., Dubois, G., Trottein, F., Fruchart-Najib, J., Mariani, J., Fruchart, J.-C. and Staels, B. (2001) EMBO Rep. 2, 42–48.
[27] Besnard, S., Heymes, C., Merval, R., Rodriguez, M., Galizzi, J.-P., Boutin, J.A., Mariani, J. and Tedgui, A. (2002) FEBS Lett. 511, 36–40.
[28] Schrader, M., Danielsson, C., Wiesenberg, I. and Carlberg, C. (1996) J. Biol. Chem. 271, 19732–19736.
[29] Karin, M. (1995) J. Biol. Chem. 270, 16483–16486.
[30] Ito, C.Y., Kazantsev, A.G. and Baldwin Jr., A.S. (1994) Nucleic Acids Res. 11, 3787–3792.
[31] Delerive, P., De Bosscher, K., Besnard, S., Vanden Berghe, W., Peters, J.M., Gonzalez, F.J., Fruchart, J.-C., Tedgui, A., Haegeman, G. and Staels, B. (1999) J. Biol. Chem. 274, 32048–32054.
[32] Chung, S.W., Kang, B.Y., Kim, S.H., Pak, Y.K., Cho, D., Trinchieri, G. and Kim, T.S. (2000) J. Biol. Chem. 275, 32681–32687.
[33] Black, B.E., Holaska, J.M., Rastinejad, F. and Paschal, B.M. (2001) Curr. Biol. 11, 1749–1758.