Rev-erbα upregulates NF-κB-responsive genes in vascular smooth muscle cells

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Abstract **Rev-erbα and retinoic acid receptor-related orphan receptor-α (RORα) are orphan nuclear receptors but their effects on transcription are opposed. Here, we show that Rev-erbα was expressed predominantly in vascular smooth muscle cells (VSMCs) rather than endothelial cells. Overexpression of Rev-erbα upregulated the expression of interleukin-6 and cyclooxygenase-2, and increased transactivation by NF-κB and translocation of p65 to the nucleus in A7r5 VSMCs. Furthermore, the expression of Rev-erbα was upregulated by RORα1 but that upregulation was attenuated by Rev-erbα itself in A7r5 VSMCs. These results suggest a regulatory link between Rev-erbα and the NF-κB pathway.**

Key words: Nuclear receptor; Nuclear factor-κB; Interleukin-6; Cyclooxygenase-2; Vascular smooth muscle cell

1. Introduction

Nuclear factor-κB (NF-κB) regulates inflammatory responses in vascular smooth muscle cells (VSMCs) by upregulating the expression of inflammatory mediators. NF-κB is a dimeric transcription factor of belonging to the Rel/NF-κB family [1,2], and activated NF-κB is present in atherosclerotic lesions [3]. Several genes upregulated in VSMCs during atherosclerosis and other inflammatory vascular diseases, including interleukin (IL) 6, IL-1, tumor necrosis factor-α (TNF-α), vascular cell adhesion molecule-1 and intracellular adhesion molecule-1, contain functional κB elements in their promoter/enhancer regions [2]. Thus, activation of NF-κB leads to transcription of these proinflammatory genes.

Rev-erbα is an orphan member of the steroid/thyroid hormone receptor superfamily [4,5]. Rev-erbα binds as a monomer to a retinoic acid receptor-related orphan receptor (ROR) response elements (RORE) composed of a 6 bp A/T-rich sequence immediately preceding a site with the core motif of RGGTCA (R = A or G) [6]. It has also been shown to bind as a homodimer to the RevDR2 response element, which is composed of a 6 bp A/T-rich sequence immediately preceding a site with a tandem repeat of two RGGTCA motifs spaced by two nucleotides [7]. Because Rev-erbα lacks a conserved carboxy-terminal activation domain, it behaves as a transcriptional repressor interacting with other nuclear receptor corepressors such as N-CoR [7,8]. Moreover, the Rev-erbα promoter region contains both a RORE and a RevDR2 element. Rev-erbα suppresses activity at its own promoter via the RevDR2 element [9], which is also essential for the upregulation of Rev-erbα by peroxisome proliferator-activated receptor-α (PPARα) [10]. Rev-erbα is expressed in a variety of tissue types, including brown fat, skeletal muscle, and liver [4,11]. Although the biological function of Rev-erbα remains unknown, it has been implicated in adipogenesis [12,13] and muscle differentiation [14].

RORα is also a member of the nuclear receptor superfamily. The human RORα gene encodes at least four distinct splicing isoforms, RORα1, α2, α3, and α4 [15]. RORα binds either as a monomer to a RORE or as a homodimer to a RevDR2 element and shows constitutive transactivational activity via these elements [11,16]. Thus, RORα binds similar response elements to Rev-erbα but the effects of Rev-erbα and RORα on transcription are opposed to each other. Interestingly, RORα1 has been shown to suppress TNF-α-induced expression of proinflammatory genes in part by inhibiting the NF-κB signaling pathway in VSMCs [17]. We had previously shown that RORα1 and RORα4 suppress TNF-α-induced expression of adhesion molecules in part by inhibiting the NF-κB signaling pathway in human umbilical vein endothelial cells (HUVECs) [18].

Since Rev-erbα and RORα are expressed in the same tissues and regulate similar target genes but in opposite direction we investigated the effects of Rev-erbα on inflammation. Here we report that Rev-erbα is expressed in vascular cells and regulates their inflammatory responses.

2. Materials and methods

2.1. Cells and materials

Human aortic VSMCs and HUVECs were maintained in SmBM-2 BulletKit and EBM-2 BulletKit, respectively (all from Clonetics, San Diego, CA, USA). A7r5 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. HepG2 cells (ATCC) were cultured in minimum essential medium Eagle containing 10% fetal bovine serum and non-essential amino acids. TNF-α was purchased from R&D Systems (Abingdon, UK).

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Abbreviations: COX-2, cyclooxygenase-2; HUVECs, human umbilical vein endothelial cells; IL, interleukin; NF-κB, nuclear factor-κB; RORα, retinoic acid receptor-related orphan receptor-α; RORE, ROR response element; PPAR, peroxisome proliferator-activated receptor; rRNA, ribosomal RNA; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells

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2.2. Plasmids

Rev-erbα cDNA was cloned from human fetal liver (Human QUICK-Clone cDNA, Clontech, Palo Alto, CA, USA) and inserted into the vector pcDNA (Invitrogen, Carlsbad, CA, USA), creating pcDNA-Rev-erbα. The constructs pTK-RORE-Luc and pcDNA-RORα1 containing RORα1 cDNA have been described previously [18]. Plasmid pTK-RevDR2-Luc was constructed by cloning three tandem copies of RevDR2 (TCCAACTAGGTCACTAGGTCAA-AG) [19] into the luciferase reporter plasmid, which contained a thymidine kinase promoter. Plasmids pNF-κB-Luc and pAP-1-Luc were obtained from Stratagene (La Jolla, CA, USA).

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

Total RNA was isolated from HUVECs, human aortic VSMCs, A7r5 cells and HepG2 cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR analysis was performed on 15 ng total RNA with the GeneAmpEZ rTth RNA PCR Kit (Applied Biosystems, Foster City, CA, USA). The specific primer sets for the target genes are as follows: for human Rev-erbα, 5′-ACTTCCCACCATCCCC-CACT-3′ and 5′-GGAAGAAGGGGAGCCGTCAT-3′; for rat Rev-erbα, 5′-AACAACCTTTTTGGCGGCTCA-3′ and 5′-TGGTGTTG-CCTTGCCGTAGA-3′; for human RORα1, 5′-CGGTGCGCAGA-CAGAGCTATT-3′ and 5′-TTGTCTCCACAGATCTTGCATGG-3′; for human and rat GAPDH, 5′-AGCTCACTGGCATGGCC-TTC-3′ and 5′-CGCCTGCTTCACCACCTTCT-3′. RT-PCR was carried out by incubating the reaction mixture for 30 min at 60°C, then 60 s at 94°C, followed by 30 or 35 cycles of 15 s at 94°C and 15 s at 58°C. RT-PCR products were analyzed by electrophoresis in a 3% agarose gel followed by ethidium bromide staining. Quantitative real-time RT-PCR was carried out essentially as described [18], using the above primer sets or the following primers: for rat IL-6, 5′-CCAGC-CAGTTGCCTTCTTGG-3′ and 5′-GCCTCCGACTTGTGAAGTG-GT-3′; for rat cyclooxygenase-2 (COX-2), 5′-CCCATGTCAAAA-CCGTGGTG-3′ and 5′-CTGTGTTTGGGGTGGGCTTC-3′. The mRNA expression levels were normalized by ribosomal RNA (rRNA) or GAPDH expression (TaqMan control reagent, Applied Biosystems) and presented as the relative expression level compared to the expression levels obtained from the control.

2.4. Cell transfection and transcriptional assay

As a measure of transcriptional activity of RORα1 and Rev-erbα, A7r5 cells were co-transfected using FuGENE6 (Roche, Basel, Switzerland) with 0.5 μg of pTK-RORE-Luc or pTK-RevDR2-Luc, mixed with 0.1–0.3 μg of pcDNA, pcDNA-RORα1 and/or pcDNA-Rev-erbα. The transfected cells were cultured for 24 h in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and then luciferase activity was measured (Dual Luciferase Assay Kit; Promega, Madison, WI, USA). To measure transactivation by NF-κB and activator protein-1 (AP-1), A7r5 cells were co-transfected using FuGENE6 with 0.5 μg of either pNF-κB-Luc or pAP-1-Luc, mixed with 0.1–0.3 μg of pcDNA, pcDNA-RORα1 or pcDNA-Rev-erbα. The transfected cells were cultured for 16 h in Dulbecco's modified Eagle's medium containing 4% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT, USA). After treatment with 1 or 10 ng/ml TNF-α for 6 h, luciferase activity was measured. In these 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. For overexpression of Rev-erbα and RORα1, A7r5 cells were transfected using FuGENE6 with 0.2 μg of pcDNA, pcDNA-Rev-erbα, and/or pcDNA-RORα1. The transfected cells were cultured for 24 h in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

2.5. Western blot analysis

Cytoplasmic and nuclear extracts were prepared from A7r5 cells 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 a specific antibody against NF-κB p65 (sc8008; 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 five experiments. Significance was determined either by Student's *t*-test for comparing two groups or by analysis of variance, followed by Dunnett's test for multiple comparisons. A value of $P < 0.05$ was considered significant.

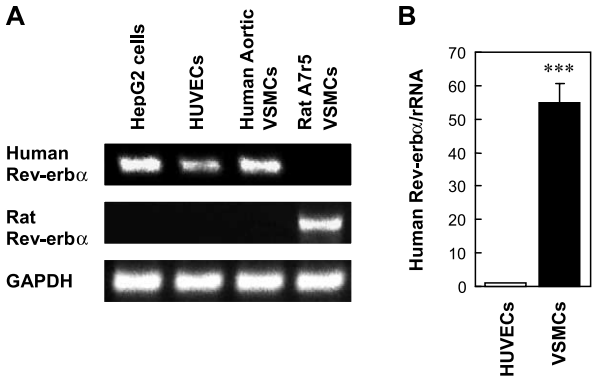

Fig. 1. A: Rev-erbα is expressed in vascular cells. Total RNA (15 ng) from HepG2 cells, HUVECs, human aortic VSMCs, and A7r5 VSMCs was analyzed by RT-PCR (35 cycles) as described. B: Rev-erbα is predominantly expressed in VSMCs. Total RNA from HUVECs and human aortic VSMCs was analyzed by quantitative real-time RT-PCR as described. The normalized expression of human Rev-erbα by rRNA in VSMCs is presented as the relative expression to the expression in HUVECs. ***$P < 0.001$ versus HUVECs.

3. Results

3.1. Rev-erbα is expressed in vascular cells

We first investigated whether Rev-erbα is expressed in vascular cells by RT-PCR. The expression of Rev-erbα mRNA in HepG2 cells was confirmed (Fig. 1A), as described previously [9,10]. We found that Rev-erbα was also expressed in HUVECs and human aortic VSMCs. Using specific primers for rat Rev-erbα, the expression of Rev-erbα was detected in rat A7r5 VSMCs. Quantitative real-time RT-PCR analysis revealed that the relative expression level of Rev-erbα/rRNA in human VSMCs was clearly higher than that in HUVECs (Fig. 1B). These data show that Rev-erbα was predominantly expressed in VSMCs compared to endothelial cells.

3.2. Rev-erbα and RORα1 show opposing transcriptional activity in A7r5 VSMCs

RORα binds to similar response elements as Rev-erbα (RORE and RevDR2 element), but the effects of Rev-erbα and RORα on transcription are opposite [7,8,11,16]. To investigate their transcriptional activity in VSMCs, we carried out transient transcriptional assay using a luciferase reporter plasmid containing RORE (4×) or RevDR2 (3×), along with an expression vector containing either Rev-erbα or RORα1. Because of the inability to transfect primary cultured human VSMCs, rat A7r5 VSMCs were used in these experiments. When the expression plasmid containing human Rev-erbα or human RORα1 was transfected into rat A7r5 cells, the ectopic overexpression of Rev-erbα or RORα1 was confirmed by RT-PCR (Fig. 2A). When the Rev-erbα expression plasmid was co-transfected with the luciferase reporter plasmid, RORE- and RevDR2-dependent luciferase activity decreased (Fig. 2B,C). In contrast, co-transfection of the RORα1 expression plasmid with the reporter plasmid led to RORE- and RevDR2-dependent luciferase activity. These results indicate that RORα1 and Rev-erbα showed opposing transcrip-

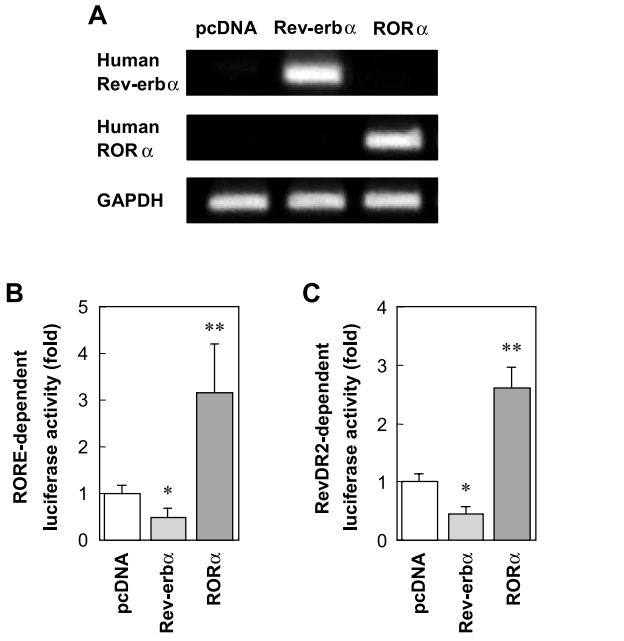

Fig. 2. A: Overexpression of Rev-erbα and RORα1 in A7r5 VSMCs. A7r5 cells were transfected with 0.1 μg of pcDNA, pcDNA-Rev-erbα, or pcDNA-RORα1. Total RNA was prepared and analyzed by RT-PCR (30 cycles) as described. B,C: Rev-erbα and RORα1 show opposing transcriptional activity in A7r5 VSMCs. pTK-RORE-Luc plasmid (B) or pTK-RevDR2-Luc plasmid (C) was transfected along with 0.1 μg of pcDNA, pcDNA-RORα1, or pcDNA-Rev-erbα. Luciferase activity was measured after 24 h. The results are presented as fold induction of the activity of pcDNA-transfected cells. $*P<0.05$, $**P<0.01$ versus pcDNA.

tional activity from the RORE and RevDR2 element in A7r5 VSMCs.

3.3. Rev-erbα induces the expression of IL-6 and COX-2 in A7r5 VSMCs

RORα1 negatively regulates TNF-α-induced proinflammatory gene expression [17,18]. Based on these reports and the opposing transcriptional activity of RORα1 and Rev-erbα, we examined the potential involvement of Rev-erbα in regulating

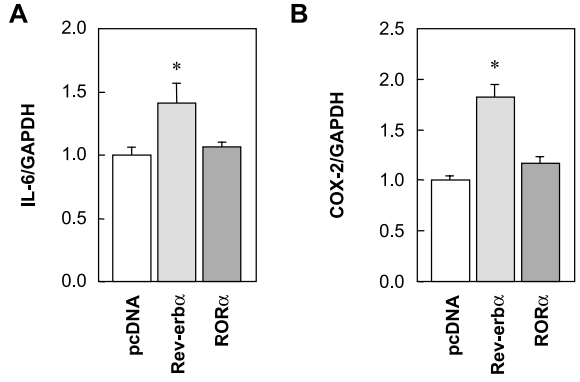

Fig. 3. Overexpression of Rev-erbα induces the expression of IL-6 and COX-2 in A7r5 VSMCs. A7r5 cells were transfected with 0.2 μg of pcDNA, pcDNA-Rev-erbα, or pcDNA-RORα1, and cultured for 24 h. Total RNA was prepared, and analyzed by quantitative real-time RT-PCR as described. The normalized expression of rat IL-6 (A) or rat COX-2 (B) to GAPDH is presented as the relative expression to the basal expression in pcDNA-transfected cells. $*P<0.01$ versus pcDNA.

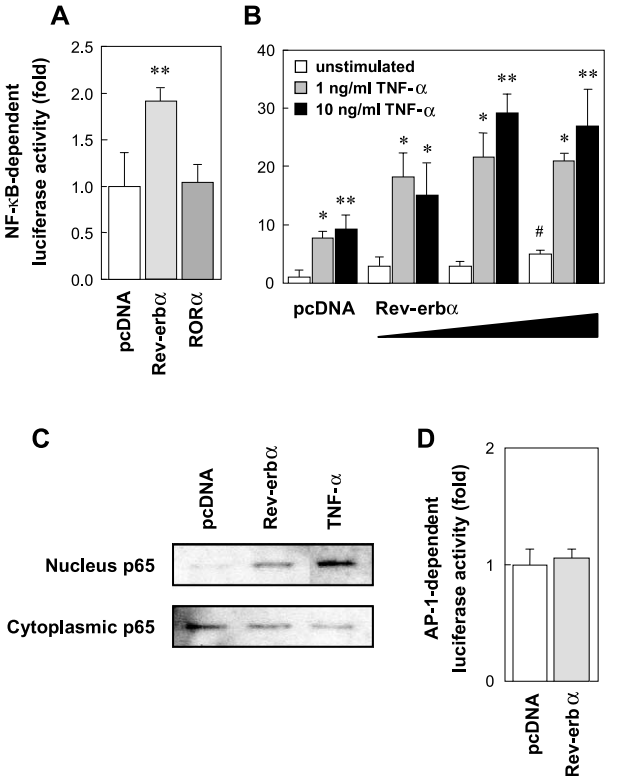

Fig. 4. Rev-erbα upregulates NF-κB activation in A7r5 VSMCs. A: A7r5 cells were co-transfected with the pNF-κB-Luc plasmid, along with 0.1 μg of pcDNA, pcDNA-Rev-erbα or pcDNA-RORα1. After 24 h, luciferase activity was measured. The results are presented as fold induction of the activity of pcDNA-transfected cells. $**P<0.01$ versus pcDNA. B: A7r5 cells were co-transfected with the pNF-κB-Luc plasmid and either pcDNA (0.3 μg) or pcDNA-Rev-erbα (0.1, 0.2 or 0.3 μg), and cultured for 16 h. Luciferase activity was measured after the treatment for 6 h with or without TNF-α as indicated. The results are presented as fold induction of the activity of pcDNA-transfected and TNF-α-unstimulated cells. $*P<0.05$, $**P<0.01$ versus TNF-α unstimulation, $^{\#}P<0.05$ versus pcDNA transfection and TNF-α unstimulation. C: A7r5 cells were transfected with 0.3 μg of either pcDNA or pcDNA-Rev-erbα, and treated with or without 1 ng/ml TNF-α for 6 h. Cytoplasmic and nuclear extracts were prepared, and then p65 was detected with the specific antibody. D: A7r5 cells were co-transfected with the pAP-1-Luc plasmid, along with 0.3 μg of pcDNA or pcDNA-Rev-erbα. After 24 h, luciferase activity was measured.

the expression of proinflammatory genes in VSMCs. We found that overexpression of Rev-erbα in A7r5 cells led to increases in the expression of both IL-6 (Fig. 3A) and also COX-2 (Fig. 3B). In contrast, overexpression of RORα1 did not affect the basal expression of IL-6 and COX-2 in A7r5 cells.

3.4. Rev-erbα upregulates transactivation by NF-κB in A7r5 VSMCs

Since NF-κB is an important transcriptional factor for regulating expression of proinflammatory genes including IL-6 and COX-2, we investigated whether Rev-erbα could upregulate transactivation by NF-κB. Co-transfection in A7r5 cells of the Rev-erbα expression plasmid and the reporter plasmid that contains NF-κB binding sites (5×) in its promoter, led to NF-κB-dependent luciferase activity (Fig. 4A). Increasing amounts of the Rev-erbα expression plasmid gave a dose-dependent upregulation of luciferase activity (Fig. 4B). Interest-

ingly, addition of TNF-α led to a potent further induction of transactivation over either basal levels or the Rev-erbα-induced levels. In contrast, co-transfection in A7r5 cells of the RORα1 expression plasmid with the reporter plasmid did not affect the luciferase activity (Fig. 4A). These results indicate that the net transcriptional response from NF-κB is influenced by the expression levels of Rev-erbα in VSMCs.

3.5. *Rev-erbα induces nuclear translocation of p65*

Next, to confirm the upregulation of NF-κB activity by overexpression of Rev-erbα, we examined the nuclear translocation of p65. NF-κB is composed of 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 expected, overexpression of Rev-erbα increased the nuclear abundance of p65 in A7r5 cells, in the same way as TNF-α treatment (Fig. 4C). Furthermore, overexpression of Rev-erbα did not affect the p65 mRNA levels in A7r5 cells (data not shown). Therefore, these data indicate that overexpression of Rev-erbα upregulates NF-κB signal activation without regulating p65 protein expression.

3.6. *Rev-erbα does not regulate transactivation by AP-1 in A7r5 VSMCs*

Since AP-1 is another important transcriptional factor in regulation of IL-6 and COX-2 expression, we tested whether Rev-erbα could regulate transactivation by AP-1. There is no significant effect on AP-1-dependent transcription when A7r5 cells were co-transfected with the Rev-erbα expression plasmid and a reporter plasmid that contains AP-1 binding sites (7×) in its promoter (Fig. 4D).

3.7. *Rev-erbα suppresses RORα1-induced Rev-erbα expression in A7r5 VSMCs*

The promoter region of Rev-erbα contains both a RORE and a RevDR2 element. The expression of Rev-erbα is upregulated by RORα1 via the RORE in L6 myoblasts [20] and via the RevDR2 element in HepG2 cells [21]. In contrast, the transcriptional activity from the promoter region of Rev-erbα is negatively regulated by Rev-erbα itself [9]. We investigated, therefore, whether the expression of Rev-erbα could be regulated either by RORα1 or by Rev-erbα itself in VSMCs. Overexpression of human RORα1 upregulated the expression of rat Rev-erbα in A7r5 cells (Fig. 5A). The upregulation was suppressed by co-transfection with the human Rev-erbα expression plasmid. However, overexpression of human Rev-erbα alone did not suppress the basal expression levels of endogenous rat Rev-erbα. Thus, the ectopic overexpression of human RORα and human Rev-erbα in rat cells leads to upregulation by RORα1 of the endogenous expression of rat Rev-erbα but the upregulation is attenuated by Rev-erbα itself.

Finally, we tested whether the cross-talk between RORα and Rev-erbα could be mediated through RORE and/or RevDR2. Co-transfection of the RORα1 expression plasmid with the reporter plasmid increased RORE-dependent luciferase activity in A7r5 VSMCs. Increasing the Rev-erbα expression plasmid with constant levels of the RORα1 expression plasmid suppressed the RORα1-induced luciferase activity (Fig. 5B). Addition of increasing amounts of the Rev-erbα expression plasmid to the RORα1 expression plasmid led to a potent suppression of the RevDR2-dependent transcription

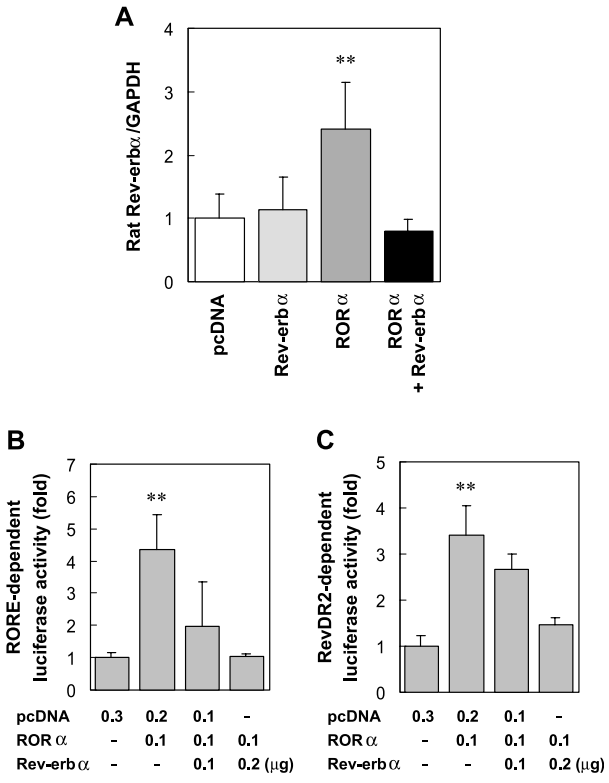

Fig. 5. A: Rev-erbα suppresses RORα1-induced Rev-erbα expression in A7r5 VSMCs. A7r5 cells were transfected with pcDNA (0.2 μg), pcDNA-Rev-erbα (0.1 μg), or pcDNA-RORα1 (0.1 μg), or co-transfected with pcDNA-RORα1 (0.1 μg) and pcDNA-Rev-erbα (0.1 μg). Total RNA was analyzed by quantitative real-time RT-PCR as described. Rat Rev-erbα expression normalized to GAPDH is presented as the relative expression to the basal expression of pcDNA. $^{**}P < 0.01$ versus pcDNA. B,C: Rev-erbα attenuated transcription by RORα1 in A7r5 VSMCs. A7r5 cells were co-transfected with either pTK-RORE Luc plasmid (B) or pTK-RevDR2-Luc plasmid (C), along with indicated amounts of pcDNA, pcDNA-RORα1, and/or pcDNA-Rev-erbα. Luciferase activity was measured after 24 h. The results are presented as fold induction of the activity of pcDNA-transfected cells. $^{**}P < 0.01$ versus pcDNA.

(Fig. 5C) in a similar dose-dependent manner to that observed with RORE-dependent transcription. These results suggest that Rev-erbα negatively regulates RORα1-induced gene expression from RORE and RevDR2 in VSMCs.

4. Discussion

Vascular endothelial cells and VSMCs express several nuclear receptors including the glucocorticoid receptor and PPAR which regulate the expression of genes involved in inflammatory responses [22,23]. Recently, RORα was shown to be expressed in vascular cells [18,24] and to suppress TNF-α-induced expression of proinflammatory genes [17,18]. However, the expression and biological functions of Rev-erbα in vascular cells remains unclear, although Rev-erbα recognizes similar response elements to RORα. In this study, we showed that Rev-erbα was predominantly expressed in VSMCs as compared to HUVECs. We therefore investigated the effect of Rev-erbα on the expression of NF-κB-responsive genes in rat A7r5 VSMCs. Our results showed for the first time that overexpression of Rev-erbα upregulates the expression of NF-κB-responsive genes such as IL-6 and COX-2 in VSMCs.

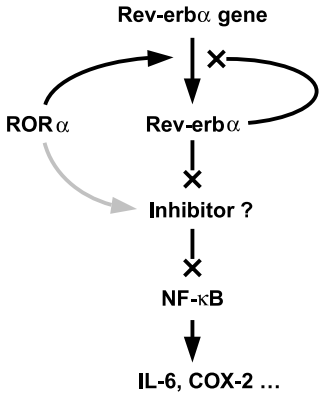

Fig. 6. Model for regulation of NF-κB-responsive gene by Rev-erbα and RORα in VSMCs. RORα stimulates Rev-erbα expression via the RORE and/or the RevDR2 element. Rev-erbα could then suppress its own expression by antagonizing RORα transcriptional activity. Rev-erbα may negatively regulate unidentified inhibitor(s) that blocks NF-κB activation and thereby induce IL-6 and COX-2 expression. RORα inhibits TNF-α-induced NF-κB activation [17,18] due to upregulation of this inhibitor.

IL-6 is produced by VSMCs, and these cells may be the primary source of circulating IL-6 during coronary artery disease. IL-6 contributes to a multitude of physiological and pathophysiological processes [25]. COX-2 has been shown to play an important role in inflammation [26,27]. Its involvement in these pathophysiological processes depends on induction of its transcription by diverse stimuli. The genes encoding IL-6 and COX-2 are known to have NF-κB and AP-1 binding sites in their promoters [2,22,25,28]. In our transient transcriptional assay, Rev-erbα did not upregulate transactivation by AP-1 but upregulated transactivation by NF-κB (Fig. 4). Overexpression of Rev-erbα increased translocation of p65 to the nucleus. These results suggest that the upregulation of IL-6 and COX-2 expression by Rev-erbα is mediated by upregulating NF-κB activity and, moreover, that Rev-erbα may upregulate the expression of other NF-κB-responsive genes. The potency of this upregulation (IL-6 and COX-2, ca. two-fold; Fig. 3, and NF-κB activation, two-fold; Fig. 4A) is not strong compared to that of pathological stimuli such as TNF-α. We clearly show, however, that TNF-α-induced NF-κB activation is potentiated by the presence of Rev-erbα, suggesting that the expression levels of Rev-erbα influence the magnitude of the inflammatory response by pathological stimuli such as TNF-α. In contrast, RORα1 overexpression did not affect the basal expression of IL-6 and COX-2 (Fig. 3) or the basal NF-κB activity in A7r5 VSMCs (Fig. 4A). Delerive et al. have reported that RORα1 overexpression inhibits TNF-α-induced NF-κB activation in rat PAC1A VSMCs but did not suppress its basal activity [17]. Taken together, these data suggest that RORα1 does not influence the basal activity of NF-κB but regulates the inflammatory response by pathological stimuli.

In this report, we describe the potential mechanism by which the NF-κB signaling pathway is upregulated by Rev-erbα. Through its suppression of transcriptional activity, Rev-erbα may negatively regulate an inhibitory molecule(s) of the NF-κB pathway, whose promoter region has RORE and/or RevDR2 elements. In contrast, RORα may upregulate the molecule(s) through its opposing transcriptional activity. In addition, PPARα may also regulate the similar inhibitory molecule(s), because PPARα also activates RevDR2-dependent transcription [10] and inhibits proinflammatory response [23]. The inhibitor could be a molecule that blocks the activity of either RIP, TRAF1 or 2, IKK, or blocks IκB degradation through upregulation of IκB expression, inhibition of IκB phosphorylation, inhibition of IκB ubiquitination or inhibition of the proteasome. We also found that Rev-erbα and RORα1 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 Rev-erbα, and also, RORα or PPARα.

Recently, opposing effects of Rev-erbα and RORα have been shown in the rat α-fetoprotein far upstream enhancer [29], rat apoAI promoter [30,31], human apoCIII promoter [32–34], and human and rat Rev-erbα [9,20,21] by transient transcriptional assay. These promoters possess a RORE and/or a RevDR2 element. Interestingly, similarly to the regulation of endogenous expression, RORα1 has been shown to regulate only the expression of Rev-erbα [20,21]. The transcriptional assay is highly sensitive because the promoter region of the reporter plasmids used contains repeated consensus elements such as four copies of RORE, or a promoter with intrinsic high-level expression such as the SV40 promoter [9] which may lead to overestimation of inhibitory effects. Therefore, we investigated the regulation of endogenous Rev-erbα in rat A7r5 VSMCs. Here, in rat A7r5 VSMCs, we demonstrated that RORα1 upregulated the endogenous expression of rat Rev-erbα and that up-regulation was then attenuated by ectopic overexpression of human Rev-erbα. These results are the first direct evidence of cross-talk between Rev-erbα and RORα1 in regulating Rev-erbα expression. Therefore, we suggest that RORα1 and Rev-erbα may both contribute to regulating gene expression of Rev-erbα and genes involved in NF-κB activation in VSMCs (Fig. 6).

In conclusion, our study demonstrates that overexpression of Rev-erbα upregulates IL-6 and COX-2 expression as well as NF-κB-dependent transcription. Furthermore, the expression of Rev-erbα was upregulated by RORα1 and that upregulation was attenuated by Rev-erbα itself in A7r5 rat VSMCs. These results provide insights into the role that Rev-erbα plays in the regulation of the inflammatory response in VSMCs.

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