

Rev-erb α upregulates NF- κ B-responsive genes in vascular smooth muscle cells

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Received 8 January 2004; accepted 20 January 2004

First published online 11 February 2004

Edited by Michael R. Bubb

Abstract Rev-erb α and retinoic acid receptor-related orphan receptor- α (ROR α) are orphan nuclear receptors but their effects on transcription are opposed. Here, we show that Rev-erb α was expressed predominantly in vascular smooth muscle cells (VSMCs) rather than endothelial cells. Overexpression of Rev-erb α upregulated the expression of interleukin-6 and cyclooxygenase-2, and increased transactivation by NF- κ B and translocation of p65 to the nucleus in A7r5 VSMCs. Furthermore, the expression of Rev-erb α was upregulated by ROR α 1 but that upregulation was attenuated by Rev-erb α itself in A7r5 VSMCs. These results suggest a regulatory link between Rev-erb α and the NF- κ B pathway.

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

1. Introduction

Nuclear factor- κ B (NF- κ B) regulates inflammatory responses in vascular smooth muscle cells (VSMCs) by upregulating the expression of inflammatory mediators. NF- κ B is a dimeric transcription factor of belonging to the Rel/NF- κ B family [1,2], and activated NF- κ B 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 κ B elements in their promoter/enhancer regions [2]. Thus, activation of NF- κ B leads to transcription of these proinflammatory genes.

Rev-erb α is an orphan member of the steroid/thyroid hormone receptor superfamily [4,5]. Rev-erb α 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-erb α 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-erb α promoter region contains both a RORE and a RevDR2 element. Rev-erb α suppresses activity at its own promoter via the RevDR2 element [9], which is also essential for the upregulation of Rev-erb α by peroxisome proliferator-activated receptor- α (PPAR α) [10]. Rev-erb α is expressed in a variety of tissue types, including brown fat, skeletal muscle, and liver [4,11]. Although the biological function of Rev-erb α remains unknown, it has been implicated in adipogenesis [12,13] and muscle differentiation [14].

ROR α is also a member of the nuclear receptor superfamily. The human ROR α gene encodes at least four distinct splicing isoforms, ROR α 1, α 2, α 3, and α 4 [15]. ROR α 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, ROR α binds similar response elements to Rev-erb α but the effects of Rev-erb α and ROR α on transcription are opposed to each other. Interestingly, ROR α 1 has been shown to suppress TNF- α -induced expression of proinflammatory genes in part by inhibiting the NF- κ B signaling pathway in VSMCs [17]. We had previously shown that ROR α 1 and ROR α 4 suppress TNF- α -induced expression of adhesion molecules in part by inhibiting the NF- κ B signaling pathway in human umbilical vein endothelial cells (HUVECs) [18].

Since Rev-erb α and ROR α are expressed in the same tissues and regulate similar target genes but in opposite direction we investigated the effects of Rev-erb α on inflammation. Here we report that Rev-erb α 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- κ B, nuclear factor- κ B; ROR α , 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

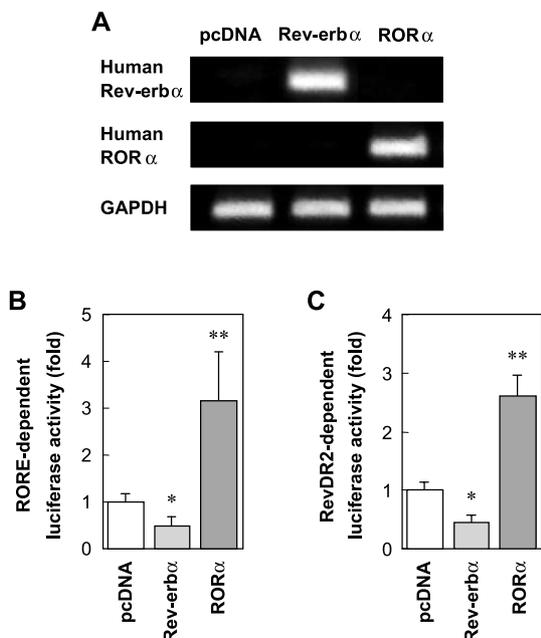


Fig. 2. A: Overexpression of Rev-erbα and RORα1 in A7r5 VSMCs. A7r5 cells were transfected with 0.1 μg of pcDNA, pcDNA-Rev-erbα, or pcDNA-RORα1. Total RNA was prepared and analyzed by RT-PCR (30 cycles) as described. B,C: Rev-erbα and RORα1 show opposing transcriptional activity in A7r5 VSMCs. pTK-RORE-Luc 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

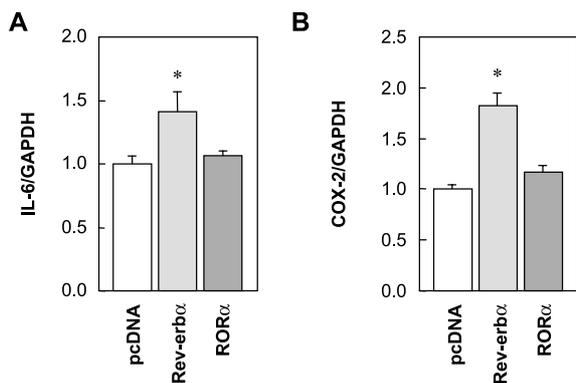


Fig. 3. Overexpression of Rev-erbα induces the expression of IL-6 and COX-2 in A7r5 VSMCs. A7r5 cells were transfected with 0.2 μg of pcDNA, pcDNA-Rev-erbα, or pcDNA-RORα1, and cultured for 24 h. Total RNA was prepared, and analyzed by quantitative real-time RT-PCR as described. The normalized expression 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.

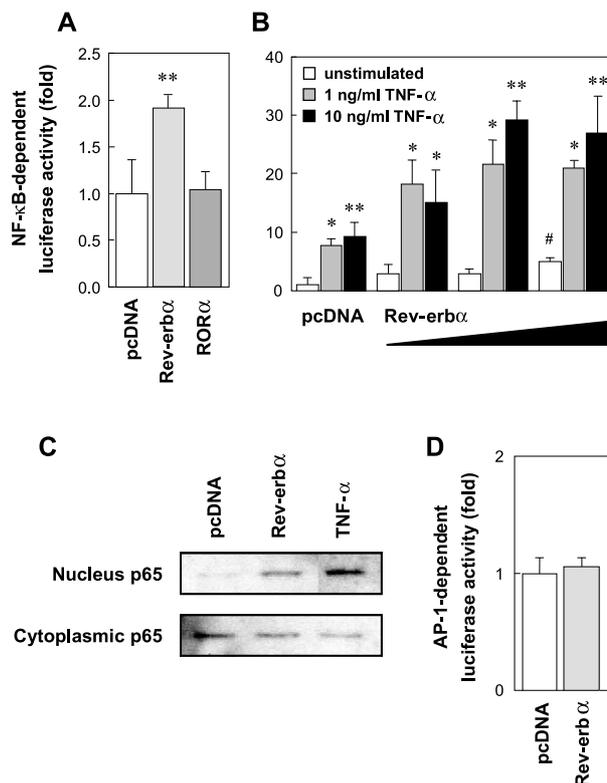


Fig. 4. Rev-erbα upregulates NF-κB activation in A7r5 VSMCs. A: A7r5 cells were co-transfected with the pNF-κB-Luc plasmid, along with 0.1 μg of pcDNA, pcDNA-Rev-erbα or pcDNA-RORα1. After 24 h, luciferase activity was measured. The results are presented as fold induction of the activity of pcDNA-transfected 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-erb α -induced levels. In contrast, co-transfection in A7r5 cells of the ROR α 1 expression plasmid with the reporter plasmid did not affect the luciferase activity (Fig. 4A). These results indicate that the net transcriptional response from NF- κ B is influenced by the expression levels of Rev-erb α in VSMCs.

3.5. Rev-erb α induces nuclear translocation of p65

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

3.6. Rev-erb α 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-erb α 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-erb α expression plasmid and a reporter plasmid that contains AP-1 binding sites (7 \times) in its promoter (Fig. 4D).

3.7. Rev-erb α suppresses ROR α 1-induced Rev-erb α expression in A7r5 VSMCs

The promoter region of Rev-erb α contains both a RORE and a RevDR2 element. The expression of Rev-erb α is upregulated by ROR α 1 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-erb α is negatively regulated by Rev-erb α itself [9]. We investigated, therefore, whether the expression of Rev-erb α could be regulated either by ROR α 1 or by Rev-erb α itself in VSMCs. Overexpression of human ROR α 1 upregulated the expression of rat Rev-erb α in A7r5 cells (Fig. 5A). The upregulation was suppressed by co-transfection with the human Rev-erb α expression plasmid. However, overexpression of human Rev-erb α alone did not suppress the basal expression levels of endogenous rat Rev-erb α . Thus, the ectopic overexpression of human ROR α and human Rev-erb α in rat cells leads to upregulation by ROR α 1 of the endogenous expression of rat Rev-erb α but the upregulation is attenuated by Rev-erb α itself.

Finally, we tested whether the cross-talk between ROR α and Rev-erb α could be mediated through RORE and/or RevDR2. Co-transfection of the ROR α 1 expression plasmid with the reporter plasmid increased RORE-dependent luciferase activity in A7r5 VSMCs. Increasing the Rev-erb α expression plasmid with constant levels of the ROR α 1 expression plasmid suppressed the ROR α 1-induced luciferase activity (Fig. 5B). Addition of increasing amounts of the Rev-erb α expression plasmid to the ROR α 1 expression plasmid led to a potent suppression of the RevDR2-dependent transcription

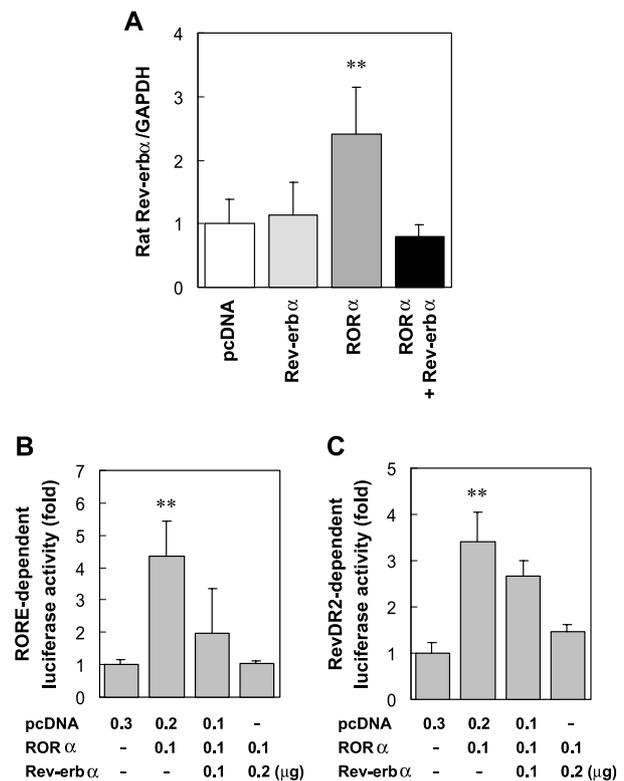


Fig. 5. A: Rev-erb α suppresses ROR α 1-induced Rev-erb α expression in A7r5 VSMCs. A7r5 cells were transfected with pcDNA (0.2 μ g), pcDNA-Rev-erb α (0.1 μ g), or pcDNA-ROR α 1 (0.1 μ g), or co-transfected with pcDNA-ROR α 1 (0.1 μ g) and pcDNA-Rev-erb α (0.1 μ g). Total RNA was analyzed by quantitative real-time RT-PCR as described. Rat Rev-erb α expression normalized to GAPDH is presented as the relative expression to the basal expression of pcDNA. ** P < 0.01 versus pcDNA. B,C: Rev-erb α attenuated transcription by ROR α 1 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-ROR α 1, and/or pcDNA-Rev-erb α . 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-erb α negatively regulates ROR α 1-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, ROR α 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-erb α in vascular cells remains unclear, although Rev-erb α recognizes similar response elements to ROR α . In this study, we showed that Rev-erb α was predominantly expressed in VSMCs as compared to HUVECs. We therefore investigated the effect of Rev-erb α on the expression of NF- κ B-responsive genes in rat A7r5 VSMCs. Our results showed for the first time that overexpression of Rev-erb α upregulates the expression of NF- κ B-responsive genes such as IL-6 and COX-2 in VSMCs.

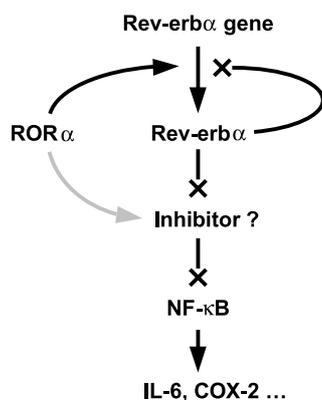


Fig. 6. Model for regulation of NF- κ B-responsive gene by Rev-erb α and ROR α in VSMCs. ROR α stimulates Rev-erb α expression via the RORE and/or the RevDR2 element. Rev-erb α could then suppress its own expression by antagonizing ROR α transcriptional activity. Rev-erb α may negatively regulate unidentified inhibitor(s) that blocks NF- κ B activation and thereby induce IL-6 and COX-2 expression. ROR α inhibits TNF- α -induced NF- κ B 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- κ B and AP-1 binding sites in their promoters [2,22,25,28]. In our transient transcriptional assay, Rev-erb α did not upregulate transactivation by AP-1 but upregulated transactivation by NF- κ B (Fig. 4). Overexpression of Rev-erb α increased translocation of p65 to the nucleus. These results suggest that the upregulation of IL-6 and COX-2 expression by Rev-erb α is mediated by upregulating NF- κ B activity and, moreover, that Rev-erb α may upregulate the expression of other NF- κ B-responsive genes. The potency of this upregulation (IL-6 and COX-2, ca. two-fold; Fig. 3, and NF- κ B 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- κ B activation is potentiated by the presence of Rev-erb α , suggesting that the expression levels of Rev-erb α influence the magnitude of the inflammatory response by pathological stimuli such as TNF- α . In contrast, ROR α 1 overexpression did not affect the basal expression of IL-6 and COX-2 (Fig. 3) or the basal NF- κ B activity in A7r5 VSMCs (Fig. 4A). Delerive et al. have reported that ROR α 1 overexpression inhibits TNF- α -induced NF- κ B activation in rat PAC1A VSMCs but did not suppress its basal activity [17]. Taken together, these data suggest that ROR α 1 does not influence the basal activity of NF- κ B but regulates the inflammatory response by pathological stimuli.

In this report, we describe the potential mechanism by which the NF- κ B signaling pathway is upregulated by Rev-erb α . Through its suppression of transcriptional activity, Rev-erb α may negatively regulate an inhibitory molecule(s) of the NF- κ B pathway, whose promoter region has RORE and/or RevDR2 elements. In contrast, ROR α may upregulate the molecule(s) through its opposing transcriptional activity. In addition, PPAR α may also regulate the similar inhibitory

molecule(s), because PPAR α 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 I κ B degradation through upregulation of I κ B expression, inhibition of I κ B phosphorylation, inhibition of I κ B ubiquitination or inhibition of the proteasome. We also found that Rev-erb α and ROR α 1 did not change the expression level of IKK β (data not shown). It would be of interest to explore further which molecule is responsible for inhibiting NF- κ B activity mediated by Rev-erb α , and also, ROR α or PPAR α .

Recently, opposing effects of Rev-erb α and ROR α 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-erb α [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, ROR α 1 has been shown to regulate only the expression of Rev-erb α [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-erb α in rat A7r5 VSMCs. Here, in rat A7r5 VSMCs, we demonstrated that ROR α 1 upregulated the endogenous expression of rat Rev-erb α and that up-regulation was then attenuated by ectopic overexpression of human Rev-erb α . These results are the first direct evidence of cross-talk between Rev-erb α and ROR α 1 in regulating Rev-erb α expression. Therefore, we suggest that ROR α 1 and Rev-erb α may both contribute to regulating gene expression of Rev-erb α and genes involved in NF- κ B activation in VSMCs (Fig. 6).

In conclusion, our study demonstrates that overexpression of Rev-erb α upregulates IL-6 and COX-2 expression as well as NF- κ B-dependent transcription. Furthermore, the expression of Rev-erb α was upregulated by ROR α 1 and that upregulation was attenuated by Rev-erb α itself in A7r5 rat VSMCs. These results provide insights into the role that Rev-erb α plays in the regulation of the inflammatory response in VSMCs.

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