

The orphan nuclear receptor ROR α is a negative regulator of the inflammatory response

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Retinoid-related orphan receptor α (ROR α) (NR1F1) is a member of the nuclear receptor superfamily whose biological functions are largely unknown. Since *staggerer* mice, which carry a deletion in the ROR α gene, suffer from immune abnormalities, we generated an adenovirus encoding ROR α 1 to investigate its potential role in control of the inflammatory response. We demonstrated that ROR α is expressed in human primary smooth-muscle cells and that ectopic expression of ROR α 1 inhibits TNF α -induced IL-6, IL-8 and COX-2 expression in these cells. ROR α 1 negatively interferes with the NF- κ B signalling pathway by reducing p65 translocation as demonstrated by western blotting, immunostaining and electrophoretic mobility shift assays. This action of ROR α 1 on NF- κ B is associated with the induction of I κ B α , the major inhibitory protein of the NF- κ B signalling pathway, whose expression was found to be transcriptionally upregulated by ROR α 1 via a ROR response element in the I κ B α promoter. Taken together, these data identify ROR α 1 as a potential target in the treatment of chronic inflammatory diseases, including atherosclerosis and rheumatoid arthritis.

INTRODUCTION

Retinoids, vitamin D, fatty acid derivatives, thyroid and steroid hormones regulate developmental and physiological processes in vertebrates by binding to specific transcription factors belonging to the nuclear receptor superfamily (Mangelsdorf *et al.*, 1995). In addition to these ligand-activated receptors, other members of the family are structurally related proteins for which no ligand has yet been identified, and therefore are referred to as 'orphan' receptors. The retinoid-related orphan receptor (ROR) subfamily contains three members, ROR α (NR1F1), ROR β

(NR1F2) and ROR γ (NR1F3). The ROR α gene generates four isoforms that share common DNA-binding domains (DBD) and putative ligand-binding domains (LBD) but are distinguished by different N-terminal domains (Carlberg *et al.*, 1994; Giguère *et al.*, 1995). ROR α isoforms bind, as monomers, to ROR response elements (RORE) composed of a 6 bp A/T rich region immediately preceding a half core AGGTCA motif (Giguère *et al.*, 1995; McBroom *et al.*, 1995; Ma *et al.*, 1998). In addition, as similarly reported for Rev-erb α , another orphan receptor ROR α is also able to bind DR-2 elements as a homodimer (Moraitis and Giguère, 1999).

Numerous binding sites for ROR α have been found within gene promoters, but the transcriptional regulation of these genes by ROR α has only been functionally demonstrated for γ F-crystallin (Tini *et al.*, 1995), N-myc (Dussault and Giguère, 1997), laminin B1 (Matsui, 1996), rat apolipoprotein AI (Vu-Dac *et al.*, 1997), Purkinje cell protein-2 (Matsui, 1997) and prosaposin (Jin *et al.*, 1998). Since ROR α regulates a wide spectrum of genes expressed in various tissues, it is difficult to assign a precise role to this orphan receptor. However, analysis of ROR α -deficient mice revealed that ROR α plays a crucial role in the development of the central nervous system (Hamilton *et al.*, 1996; Dussault *et al.*, 1998; Steinmayr *et al.*, 1998). ROR α -deficient mice display the cerebellar defects of the ataxic *staggerer* mice: they suffer from impaired motor coordination, hanging and equilibrium deficits. Homozygous *staggerer* mice carry a deletion within the ROR α gene that prevents the translation of the ligand-binding domain (Hamilton *et al.*, 1996). Interestingly, the premature death of the *staggerer* mice does not correlate with the cerebellar defects, suggesting that ROR α may play additional roles in development and physiology.

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RESULTS AND DISCUSSION

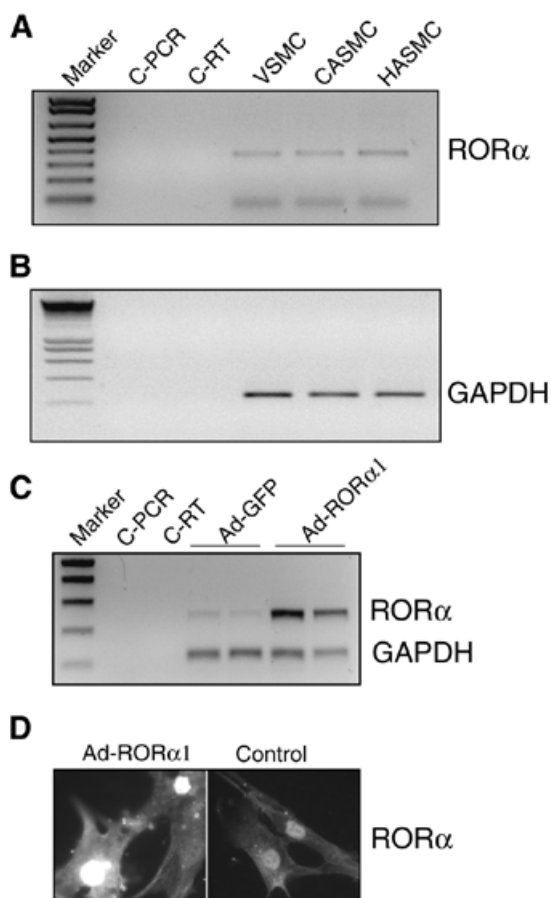


Fig. 1. ROR α is expressed in different vascular SMC types. (A and B) RT-PCR (35 cycles) analysis of ROR α and GAPDH mRNA. C-PCR and C-RT are negative controls for PCR and RT, respectively; VSMC, smooth muscle cells from saphenous veins; CASMC, human coronary artery smooth muscle cells; HASMC, human aortic smooth muscle cells. (C) RT-PCR (30 cycles) analysis of ROR α mRNA in SMC infected with Ad-ROR α 1 or Ad-GFP for 24 h. (D) Analysis of ROR α protein expression in SMC infected for 24 h with or without Ad-ROR α 1. Immunocytochemistry experiments were performed as previously described (Chinetti et al., 1998) using a rabbit polyclonal ROR α antibody raised against aa 163–225.

Since ROR α may play a role in inflammation (Kopmels et al., 1992) and atherosclerosis (Mamontova et al., 1998), we first examined ROR α expression in human primary SMC by RT-PCR analysis. ROR α expression was readily detected in the different vascular SMC types analysed (Figure 1A). To investigate a potential role of ROR α in the control of the inflammatory response, we generated by homologous recombination an adenovirus encoding the ROR α 1 isoform (Ad-ROR α 1), which is the predominantly expressed transcript among the four isoforms (Forman et al., 1994). Infection of primary SMC with Ad-ROR α 1 led to a strong expression of ROR α mRNA levels whereas infection with a control Ad-GFP did not affect endogenous levels (Figure 1C). Immunocytochemistry experiments using a specific polyclonal antibody raised against ROR α (aa 163–225) confirmed these results at the protein expression level and further demonstrated that both endogenous and exogenous ROR α are principally localized in the nucleus (Figure 1D).

Next, the influence of ROR α 1 on different markers of the inflammatory response to TNF α was tested. In absence of stimulation, both IL-6 and IL-8 were detected at low levels in the culture medium of Ad-GFP-infected cells. This low cytokine production was significantly repressed in ROR α 1-infected cells (Figure 2A and B). TNF α treatment of Ad-GFP-infected cells for 24 h led to a sharp induction of both cytokines, which was significantly repressed by ROR α 1 infection (Figure 2A and B). COX-2 protein levels were monitored by western blot analysis in the same experiments. TNF α strongly induced COX-2 expression, which was completely blunted in Ad-ROR α 1-infected cells (Figure 2C). Finally, in Ad-GFP-infected cells, we failed to detect endogenous ROR α protein, which is likely to be due to the low affinity of our antibody. However, in Ad-ROR α 1 cells, high amounts of ROR α protein were detected and its levels were not affected by TNF α stimulation (Figure 2D). These data indicate that ROR α 1 negatively regulates the TNF α -induced inflammatory response in primary aortic SMC.

Since the IL-6, IL-8 and COX-2 genes have been reported to be NF- κ B-driven genes (Thurberg and Collins, 1998), we hypothesized that ROR α 1 may negatively interfere with the NF- κ B signalling pathway. Because of the inability to transfect primary cultured human aortic SMC, rat SMC PAC1A cells were transiently transfected with an NF- κ B-driven promoter construct in the presence or absence of ROR α 1. Since these cells were unresponsive to TNF α stimulation, lipopolysaccharide (LPS) was used as an NF- κ B signalling inducer. LPS treatment significantly (>3-fold) increased NF- κ B-dependent promoter activity. This induction was abolished by ROR α 1 cotransfection whereas basal promoter activity was not significantly affected (Figure 3A). These results suggest that ROR α 1 may exert its anti-inflammatory activities by inhibiting NF- κ B transcriptional activity.

To obtain further insight into the molecular mechanisms of the anti-inflammatory activities of ROR α 1, we next tested, by western blot analysis and by immunocytochemistry experiments, the influence of ROR α 1 overexpression on p65 nuclear translocation, a critical event in NF- κ B signaling pathway activation. As expected, TNF α treatment for 30 min led to p65 nuclear translocation in Ad-GFP-infected cells (Figure 3B and C). This translocation was less pronounced in Ad-ROR α 1-infected cells

Since it has been demonstrated that *staggerer* mice fed a high-fat diet develop severe atherosclerosis (Mamontova et al., 1998), and since these mice display immune abnormalities associated with increased mRNA levels of IL-1 β , IL-6 and TNF α (Kopmels et al., 1992), we studied ROR α expression in primary smooth-muscle cells (SMC) and examined whether ROR α regulates the inflammatory response in these cells. Using an adenovirus encoding for ROR α 1, we demonstrate that ROR α 1 negatively regulates the inflammatory response by interfering with the NF- κ B signalling pathway. This action of ROR α 1 on NF- κ B is likely to be due to the induction of I κ B α , the major inhibitory protein of the NF- κ B signalling pathway, whose expression was found to be transcriptionally upregulated by ROR α 1 via a RORE in the I κ B α promoter.

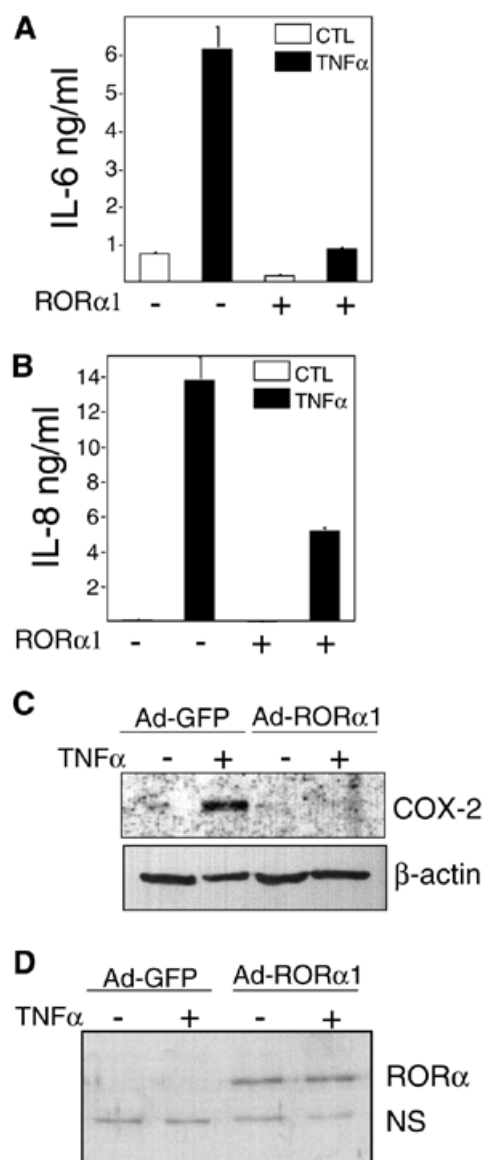


Fig. 2. RORα1 inhibits TNFα-induced IL-6, IL-8 secretion and COX-2 expression in SMC. Primary SMC were infected with Ad-RORα1 or Ad-GFP for 16 h and subsequently stimulated for 24 h with TNFα (10 ng/ml). At the end of the treatment period, IL-6 and IL-8 concentrations in the culture medium were determined by ELISAs (R&D systems, UK) (A and B), and COX-2 and RORα protein expression was measured by western blot analysis (C and D). COX-2 protein levels were normalized against cellular β-actin protein content. (NS: non-specific).

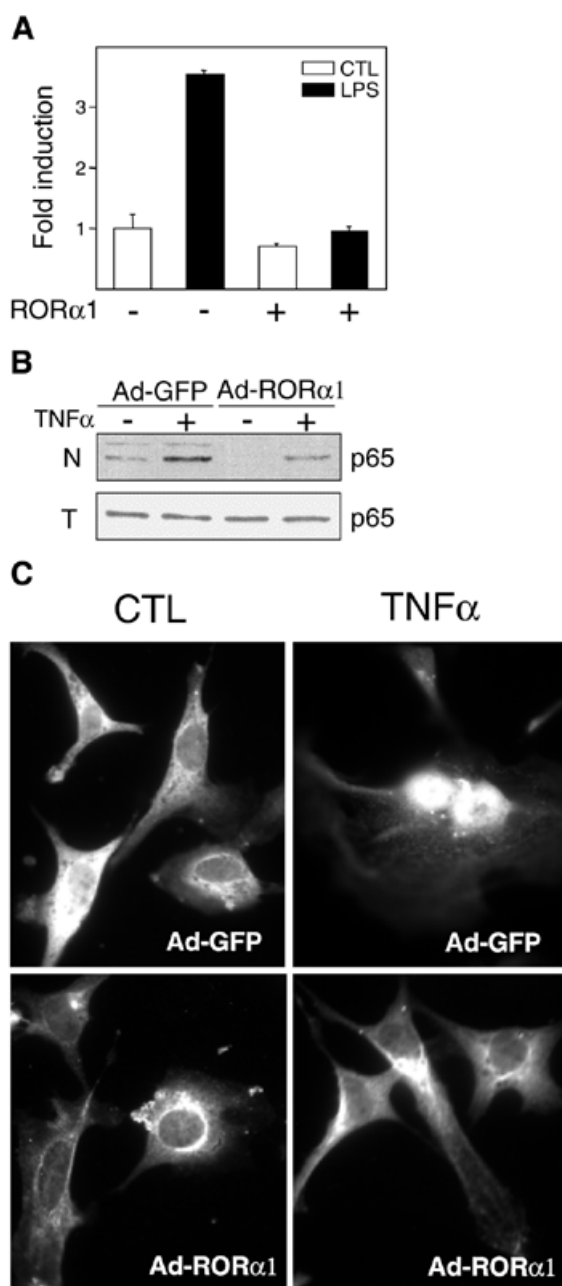


Fig. 3. RORα1 inhibits NF-κB activation. (A) PAC1A cells were transfected with the (NF-κB)₃-Luc reporter construct (100 ng) in the presence of pSG5-RORα1 (50 ng) or empty vector (pSG5 50 ng). Two hours after transfection cells were re-fed with Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2% fetal calf serum (FCS) in the presence or absence of LPS (10 μg/ml) for 24 h. (B) Primary SMC were infected with Ad-RORα1 or Ad-GFP for 16 h and subsequently stimulated for 30 min with TNFα (10 ng/ml). Total (T, lower gel) and nuclear (N, upper gel) protein extracts were prepared and subjected to western blot analysis using a p65 antibody (Santa Cruz). (C) Primary SMC were infected with Ad-RORα1 or Ad-GFP for 16 h and subsequently stimulated for 30 min with TNFα (10 ng/ml). Cells were subsequently immunostained using a rabbit polyclonal p65 antibody (Santa Cruz, sc-109).

(Figure 3B and C), suggesting that RORα1 may control p65 translocation without regulating total p65 protein expression (Figure 3B, lower panel). To explore the consequences of this reduced translocation, electrophoretic mobility shift assays (EMSA) were performed. Twenty-four hours after cell infections, TNFα was added for 30 min, and nuclear extracts were prepared and subjected to EMSA using an NF-κB radiolabelled consensus probe. Ad-GFP-infected cells expressed a basal NF-κB DNA-binding activity, which was significantly lowered in Ad-RORα1-infected cells, consistent with our previous results (Figure 2A

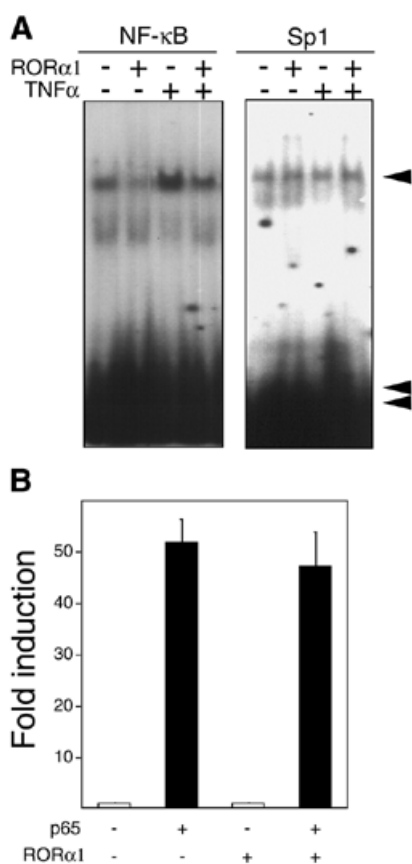


Fig. 4. ROR α 1 inhibits NF- κ B DNA-binding activities without affecting p65-mediated NF- κ B transcriptional activation. **(A)** Primary SMC were infected with Ad-ROR α 1 or Ad-GFP for 16 h and subsequently stimulated for 30 min with TNF α (10 ng/ml). Nuclear protein extracts (5 μ g) were prepared and subjected to EMSA using an NF- κ B and a Sp1 consensus radiolabelled probe (Promega). The shifted complexes are indicated by an arrowhead. A double arrowhead indicates the free probe. **(B)** PAC1A cells were transfected with the (NF- κ B) $_3$ -Luc reporter construct (100 ng) in the presence of pSG5-ROR α 1 (50 ng) and RSV-p65 (50 ng) or empty vectors (pSG5, 50 ng; RSV, 50 ng).

and B). As expected, TNF α stimulation led to a strong increase in NF- κ B DNA binding, which was again sharply reduced in Ad-ROR α 1 cells (Figure 4A). The presence of p65 protein in the NF- κ B complex was verified by shifting the complex using an anti-p65 antibody (data not shown). As a control, neither TNF α nor ROR α 1 overexpression affected Sp1 DNA binding (Figure 4A), indicating that ROR α 1 specifically interferes with NF- κ B DNA-binding activities. Since many nuclear receptors have been reported to inhibit NF- κ B-dependent transcription by physical interaction with the p65 subunit (Göttlicher *et al.*, 1998), we next explored the existence of such a mechanism. The influence of ROR α 1 co-transfection on transactivation of an NF- κ B response element-driven reporter vector by transfected p65 was tested in PAC1A cells. As expected, p65 transfection resulted in a strong induction of reporter activity (Figure 4B). However, this induction was not affected by ROR α 1 co-transfection, indicating that the negative interference of ROR α 1 with NF- κ B signalling is independent of direct association with p65

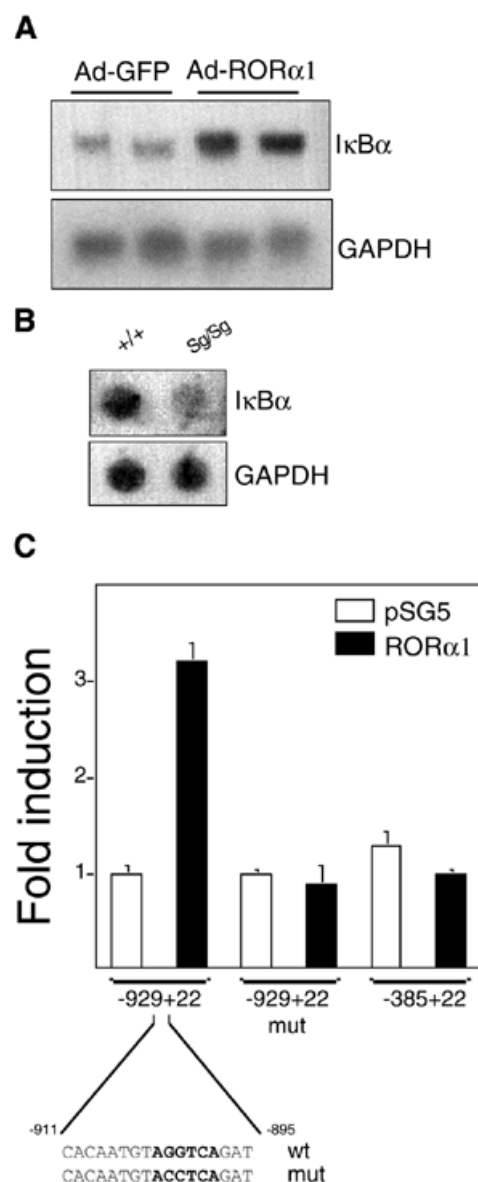


Fig. 5. ROR α 1 activates I κ B α transcription. **(A)** Primary SMC were infected with Ad-ROR α 1 or Ad-GFP for 16 h. Total RNA was prepared and I κ B α and GAPDH mRNA levels were measured by northern blot analysis. **(B)** Aortas from wild-type and *Staggerer* mice were isolated and total RNA was prepared. I κ B α and GAPDH mRNA levels were measured by dot-blot analysis. **(C)** PAC1A cells were transfected with various I κ B α promoter fragments (100 ng) in the presence of pSG5-ROR α 1 or empty vector (pSG5, 50 ng).

and thus occurs upstream of p65. Similar results were obtained with p50 co-transfection (data not shown).

In resting cells, I κ B α sequesters p50-p65 heterodimers in a cytoplasmic inactive complex. Since ROR α 1 inhibits p65 translocation and subsequent NF- κ B DNA-binding activity, I κ B α expression levels were analysed in both Ad-GFP- and Ad-ROR α 1-infected cells. Ad-GFP-infected cells express low I κ B α mRNA levels (Figure 5A). Interestingly, I κ B α transcript levels were drastically increased in Ad-ROR α 1-infected cells (Figure 5A), suggesting that ROR α 1 inhibits NF- κ B signalling by

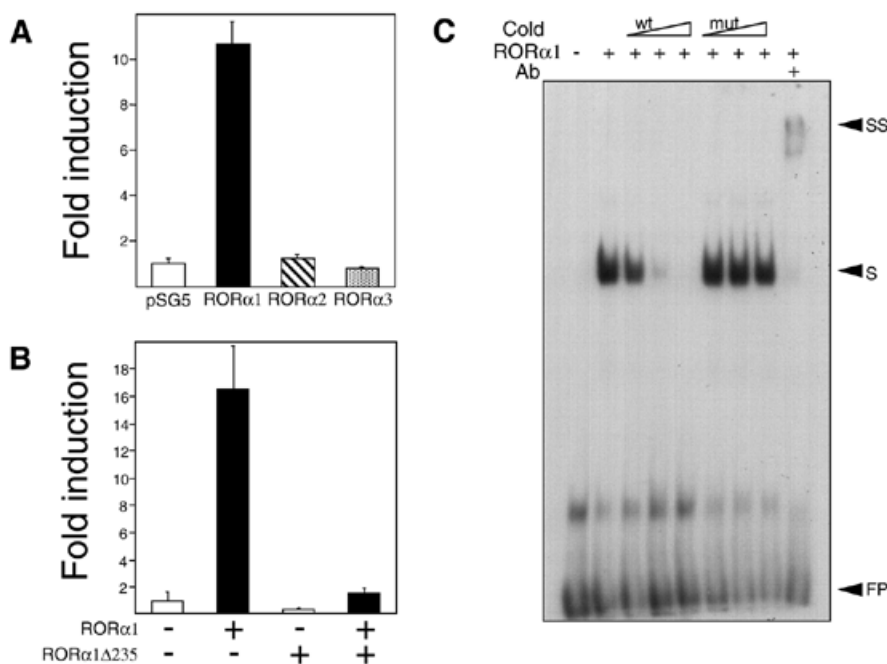


Fig. 6. RORα1, but not RORα2 nor RORα3, activates IκBα transcription and binds to the ROR site within IκBα promoter. (A) PAC1A cells were transfected with the (IκBα-RORE)₂-TK-Luc reporter vector (100 ng) in the presence of RORα1, RORα2 or RORα3 (50 ng), or empty vector (pSG5, 50 ng). (B) PAC1A cells were transfected with the (IκBα-RORE)₂-TK-Luc reporter vector (100 ng) in the presence of RORα1 (50 ng) and RORα1Δ235 or empty vector (50 ng). (C) EMSA was performed using *in vitro* translated RORα1 protein (Promega) and a radiolabelled IκBα-ROR probe. Increasing concentrations (1×, 10× and 100×) of competitor wild-type (AGGTCA) or mutated (ACCTCA) oligonucleotides were used to demonstrate the specificity of the shifted complex. To supershift the complex, 1 μl of RORα antibody was added to the binding reaction. (S, shifted complex; SS, supershifted complex; FP, free probe).

inducing IκBα gene expression. In order to provide genetic evidence for a role of RORα in the control of IκBα gene expression, IκBα mRNA levels were analysed in aortas from wild-type and homozygous *staggerer* mice by dot-blot analysis (Figure 5B). Aortas from *staggerer* mice display significantly lower basal levels of IκBα mRNA than *in vivo* wild-type mice, indicating that RORα regulates IκBα gene expression in the vascular wall (Figure 5B). Furthermore, *staggerer* mice displayed an exacerbated inflammatory response, as demonstrated by hyperproduction of IL-6 after phorbol ester treatment of splenocytes isolated from *staggerer* mice, compared with those from wild-type mice (see Supplementary data, available at *EMBO reports* Online). To further study IκBα gene regulation by RORα1, a (−929 +22) promoter fragment was PCR amplified and inserted upstream of the luciferase gene (Ito *et al.*, 1994). Co-transfection of RORα1 induced 3-fold the (−929 +22) but not the (−385 +22) promoter construct (Le Bail *et al.*, 1993), indicating that the regulatory region locates between −929 and −385 (Figure 5C). Sequence analysis revealed the presence of a putative RORE located between −911 and −895 (Figure 5C). Mutation of this putative RORE site conferred unresponsiveness to RORα1 (Figure 5C), indicating that this site mediates IκBα transcriptional induction by RORα1.

Since the RORα gene generates several isoforms (Giguère *et al.*, 1994), we next evaluated the influence of RORα1, RORα2 and RORα3 on a (IκBα-RORE)₂-driven promoter construct. As expected, RORα1 strongly induced the reporter construct activity (almost 10-fold) (Figure 6A), showing that the IκBα-

RORE can function in a promoter-independent manner. By contrast, RORα2 and RORα3 failed to induce this latter construct (Figure 6A), suggesting that IκBα transcriptional induction is specific to the RORα1 isoform. A dominant-negative RORα1Δ235 lacking the LBD was found to inhibit RORα1 transcriptional activity by competing for the same DNA-binding site (McBroom *et al.*, 1995). The effect of this mutant was next tested on RORα1-induced (IκBα-RORE)₂-driven promoter activity. As expected, co-transfection of RORα1 led to a strong induction of the promoter activity, which was completely abolished by RORα1Δ235 cotransfection (Figure 6B), suggesting that RORα1 binding to the promoter is essential for the transcriptional induction. Finally, RORα1 binding to the identified RORE site was verified by EMSA using *in vitro* translated RORα1 proteins (Figure 6C). Competition experiments using wild-type and mutated cold oligonucleotides, as well as supershift experiments, demonstrate that RORα1 binds strongly to the wild-type but not to the mutated RORE site.

In this study, we report that the overexpression of RORα1 in human aortic SMC prevents TNFα-induced IL-6, IL-8 and COX-2 expression, three important markers of the inflammatory response. RORα1 negatively regulates the cytokine-induced inflammatory response by upregulating IκBα, the major inhibitor of the NF-κB signalling pathway, at the transcriptional level, thereby reducing p65 nuclear translocation. Previous studies reported that *staggerer* mice display immune abnormalities such as IL-1β hyperproduction in macrophages (Kopmels *et al.*, 1990, 1992; Vernet-der Garabedian *et al.*, 1998). Interestingly, we

found that *staggerer* mice express lower levels of I κ B α transcript in the vascular wall compared with wild-type mice (Figure 5B). In addition, cotransfection of a dominant-negative form of ROR α abolished ROR α 1-induced I κ B α transcription (Figure 6B). These results may, at least in part, explain the inflammatory phenotype of the *staggerer* mice. Interestingly, 5-lipoxygenase, an important enzyme involved in the control of allergic and inflammatory reactions, has been reported to be a ROR α target gene (Steinhilber *et al.*, 1995). 5-lipoxygenase was shown to be downregulated by ROR α and RZR α in human B lymphocytes. ROR α 1, but not ROR α 2 nor ROR α 3, efficiently binds to RORE site in its promoter. However, this study was based on the use of melatonin as a specific ROR α activator (Becker-André *et al.*, 1994), data which are controversial (Becker-André *et al.*, 1997). Altogether, the results of the present study provide a novel function for ROR α 1 as a negative regulator of the vascular inflammatory response, as well as a potential molecular basis for its anti-inflammatory activity. The discovery of synthetic ROR α ligands may lead to the development of compounds useful in the treatment of inflammatory disorders.

METHODS

Cell culture. Primary human aortic (HA) and coronary artery (CA) SMC (PromoCell, Heidelberg, Germany) and primary SMC from saphenous veins (VSMC: a kind gift of Dr Walsh, Boston, MA) were cultured under standard conditions. Cells from passages 5–8 were used for the experiments.

RNA analysis. RNA preparation and northern blot hybridizations were performed as previously described (Staels *et al.*, 1992) using I κ B α and GAPDH cDNA fragments as probes. For RT-PCR analysis of ROR α expression, total RNA was reverse transcribed and subsequently amplified by PCR using the following primers: for ROR α , 5'-GTCAGCAGCTTCTACCTGGAC-3' and 5'-GTGTTGTTCTGAGAGTGAAAGGCACG-3' (fragment size: 482 bp); for GAPDH, 5'-ATGCAGCCCCGAATGCTCCTCATCGTGGCC-3' and 5'-TTCTTGGAGGCCATGTGG GCCAT-3' (fragment size: 239 bp).

Adenovirus generation. The recombinant adenovirus (Ad-GFP and Ad-ROR α 1) was obtained by homologous recombination in *Escherichia coli* (Chartier *et al.*, 1996) after insertion of the cDNAs into pAdCMV2 vector. Viral stocks were then created as previously described (Sardet *et al.*, 1995). Viral titres were determined by a plaque assay on 293 cells and defined as pfu/ml. Cells were infected at an input multiplicity (MOI) of 100 virus particles per cell, by adding virus stocks directly to the SMC culture medium.

Plasmids and transient transfections. The expression vectors pSG5-ROR α 1, -ROR α 2, -ROR α 3 and -ROR α 1 Δ 235 were derived from the previously reported pCMX vectors (McBroom *et al.*, 1995). The p65 and p50 expression plasmids were as previously described (Staels *et al.*, 1998). A -929+22 I κ B α promoter fragment was amplified using human genomic DNA and inserted into pGL2 basic vector (Promega) yielding p(-929+22) I κ B α -Luc. A mutation in the I κ B α promoter ROR site was introduced using the site-directed mutagenesis kit (Stratagene), leading to the mutation of the ROR site AGGTCA to ACCTCA. The p(-385+22) I κ B α -Luc reporter vector was generously provided by Dr Israël (Institut Pasteur, Paris, France). The (I κ B α -RORE)₂-TK-Luc reporter vector was generated by inserting two copies of the I κ B α ROR site in

front of the minimal TK promoter. The (NF- κ B)₃-Luc reporter construct was provided by Stratagene. PAC1A cells (a rat aortic SMC line) were transfected using a lipid-cationic technique as previously described (Delerive *et al.*, 2000). Phosphoglycerate kinase (PGK) β -galactosidase expression plasmid (50 ng) was cotransfected as a control for transfection efficiency. All transfection experiments were repeated at least three times.

Immunocytochemistry experiments. Immunostaining was performed as previously described (Chinetti *et al.*, 1998), using specific antibodies raised against ROR α and p65 (Santa Cruz, sc-109). Proteins were visualized using secondary rhodamine-conjugated anti-rabbit antibodies with a LEITZ DMR fluorescence microscope.

Supplementary data. Supplementary data for this paper are available at *EMBO reports* Online.

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