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Expression and regulation of the nuclear receptor RORα in human vascular cells

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Abstract Retinoic acid receptor-related orphan receptor α (RORα) is a member of the nuclear receptor superfamily. Using RT-PCR, RORα mRNA was identified in human aortic smooth muscle cells (hASMC), endothelial cells (EC), as well as in human mammary arteries and atherosclerotic plaques. We found a predominant expression of RORα1 in hASMC, and RORα4 in EC. RORα2 and RORα3 were not detected. In arteries, RORα4 was predominant compared with RORα1. In atherosclerotic plaques, RORα expression was significantly decreased. In hASMC stimulated with cytokines, RORα expression was increased by 2.5-fold. RORα mRNA was also significantly increased (~2-fold) in hASMC and EC cultured under hypoxia.

Key words: Nuclear receptor; Smooth muscle cell; Endothelial cell; Cytokine; Hypoxia

1. Introduction

RORα (retinoic acid receptor related orphan receptor α) is a member of the nuclear hormone-receptor superfamily [1]. Its primary sequence associates it with the class of retinoic acid receptors [2]. The characteristic structure of the nuclear receptor comprises a binding domain for the ligand (LBD), another one for the DNA (DBD), a hinge region linking both of them, and a fixation site for a modulator on the N-terminal region. The alternative splicing of the gene gives four isoforms of RORα, differing only in the modulator site [2]. This specificity enables each isoform of RORα to recognize a particular responsive element, though their DBDs are identical [3].

A spontaneous mutation consisting of a 122 bp deletion in the LBD in the RORα gene has been identified in mice [1]. The homozygous mutant mouse (sg/sg) shows cerebellar ataxia and neurodegeneration [4,5], and exhibits immune abnormalities [6]. We previously reported that the homozygous sg/sg mutant mouse fed a high-fat diet shows increased atherosclerotic lesions compared with the wild type C57BL/6 mouse [7], suggesting an important role of the nuclear receptor RORα in atherogenesis. Recently, we found increased ischemia-induced angiogenesis in sg/sg mice [8]. RORα has been reported to be expressed in human aortic smooth muscle cells (hASMC) [9], but little is known regarding its regulation in endothelial (EC) and smooth muscle cells (SMC). We therefore studied the expression of RORα isoforms in human EC and SMC, as well as in human arteries, and we investigated the regulation of RORα by cytokines and hypoxia. Our results show a constitutive RORα mRNA expression in hASMC and EC, as well as in human mammary arteries and atherosclerotic plaques. We found a predominant expression of RORα1 in hASMC and RORα4 in EC, while no expression of RORα2 or RORα3 mRNA were detected. In normal arteries, RORα4 was predominant compared with RORα1, but RORα mRNA expression was significantly decreased in atherosclerotic lesions.

In both hASMC and EC, we found that RORα was positively regulated by inflammatory cytokines.

2. Materials and methods

2.1. Cells

2.1.1. hASMC. hASMC from Clonetics (San Diego, CA, USA) were obtained from two donors. Cells were used between passages 4 and 8 and were characterized by morphological criteria and smooth muscle α-actin expression. hASMC were grown in culture medium supplemented with fetal calf serum (FCS; 5%), insulin (5 μg/ml), recombinant human basic fibroblast growth factor (FGF; 2 ng/ml), recombinant human epidermal growth factor (EGF; 10 ng/ml), gentamycin (50 μg/ml) and amphotericin B (50 ng/ml). hASMC were cultured into 25 cm^2 flasks at 3500 cells/cm^2 until subconfluence was obtained after 7 days in culture.

2.1.2. EC. Human EC were obtained from Promocell (Heidelberg, Germany). Cells were used between passages 2 and 4. EC were grown in culture medium supplemented with FCS (5%), insulin (5 μg/ml), recombinant human basic FGF (1 μg/ml), recombinant human EGF (0.1 μg/ml), gentamycin (50 μg/ml) and amphotericin B (50 μg/ml). EC were cultured into 0.2% gelatin-coated 25 cm^2 flasks at 5000 cells/cm^2 until subconfluence was obtained after 7 days in culture.

2.2. Mammary arteries and atherosclerotic plaques

Twenty atherosclerotic plaques removed from patients undergoing carotid endarterectomy were collected. For controls, four internal mammary arteries free of atherosclerosis were obtained during coronary bypass surgery. They were rapidly immersed in liquid nitrogen and stored at −80°C.

2.3. Cell stimulation

Cells were incubated from 15 min to 72 h in the presence of proinflammatory cytokines tumor necrosis factor α (TNFα; 10 U/ml), interleukin-1β (IL-1β; 10 ng/ml), and lipopolysaccharide (LPS; 10 μg/

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ml), used alone or in combination. Another set of cells was incubated during 6 h with increasing concentration of IL-1β, from 0 to 10 ng/ml. In an additional series of experiments, cells were submitted to hypoxia (95% N_2, 5% CO_2) for 3 or 6 h.

At the end of each treatment, cells were washed with iced PBS and frozen at −80°C.

IL-1β was from Genzyme, Cambridge, MA, USA; LPS from Sigma, Saint Quentin, France; TNFα from R&D, Abingdon, UK.

2.4. Determination of RORα mRNA expression

2.4.1. Total RNA isolation. Total RNA was extracted from cells and tissues, according to the Trizol reagent protocol (Life Technologies). The quality of RNA was confirmed by ethidium bromide staining in 1% agarose gel.

2.4.2. RT-PCR protocol. RORα mRNA was amplified using primers specific of the common part of the four isoforms of RORα. First-strand cDNA was performed on 50 ng of total RNA. PCR conditions were: 39°C, 60 min; 94°C, 3 min; 94°C, 45 s; 55°C, 1 min, 72°C, 1 min. The PCR fragments were visualized on a 5% polyacrylamide gel. Primers and annealing temperatures used were: RORα, forward primer 5′-GTCAGCAGCTTCTACCTGGAC-3′ and reverse primer 5′-GTGTTGTTCTGAGAGTGAAAGGCACG-3′, 25 cycles, 55°C; GAPDH forward primer 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and reverse primer 5′-GTGTTGTTCTGAGAGTGAAAGGCACG-3′, 26 cycles, 60°C.

2.4.3. RT-PCR protocol for RORα isoforms. First-strand cDNA was performed on 1 μg of total RNA. PCR conditions were: 37°C, 60 min; 94°C, 3 min; 94°C, 45 s; 60°C, 1 min, 72°C, 1 min. After 35 cycles, the PCR fragments were separated on a 0.1% agarose gel, and then blotted onto a nylon sheet. Specific band was detected with a radiolabeled probe. Primers and annealing temperatures used were: RORα1, primer 5′-AAACATGGAGTCAGCTCCG-3′; RORα2 primer 5′-CTCCAAATACTCCATCAGTGTATCC-3′; RORα3 primer 5′-CAACTTGAGCACATAAACTGG-3′; RORα4 primer 5′-GCACCGCGCTTAAATGATGT-3′. For the four isoforms, the same reverse primer 5′-CATACAAGCTGTCTCTCTGC-3′ was used.

Specific probes used for the southern analysis were: RORα1: 5′-CTTCTACCTGGACATACAGC-3′; RORα2: 5′-GGGATGAACTTTTTGGGATTC-3′; RORα3: 5′-AGAAGCTCTTCAACCTGTAG-3′; RORα4: 5′-GTGATCGCAGAGATGAAAGC-3′.

2.5. Data analysis

For RT-PCR, percent expression of RORα was expressed relative to GAPDH mRNA signal. Results are expressed as mean ± S.E.M. Data were compared by the use of a one-way ANOVA. A $P < 0.05$ value was considered statistically significant.

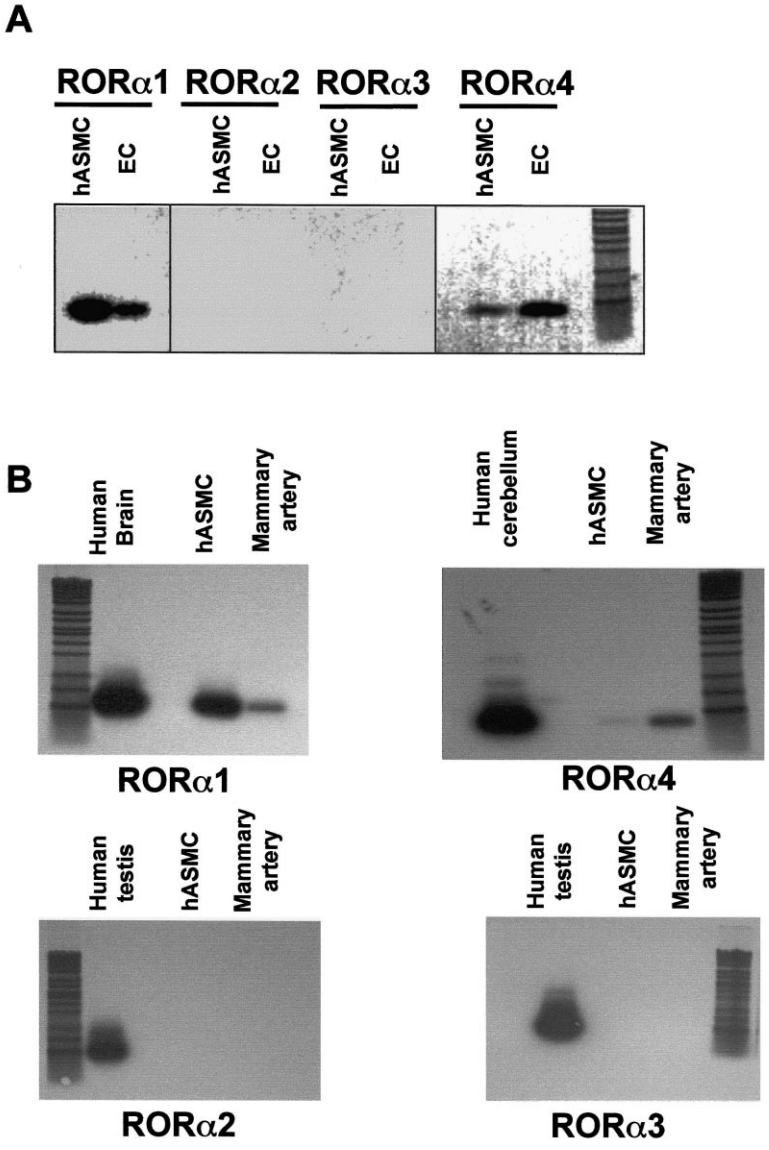

Fig. 1. A: RORα isoforms expression in human vascular cells. B: RORα isoforms expression in human SMC and normal arteries.

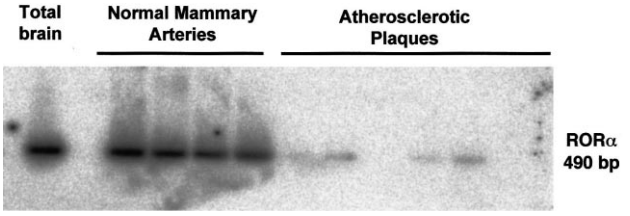

Fig. 2. RORα mRNA expression in human normal or atherosclerotic arteries. Atherosclerotic plaques were obtained from carotid endarterectomia. 50 ng of total RNA was assayed for 25 cycles for RORα and 26 cycles for GAPDH. Amplified products are electrophoresed onto a 5% polyacrylamide gel.

3. Results

We first verified whether RORα was expressed in human vascular cells and arteries. RORα mRNA was found to be constitutively expressed in both hASMC and EC, as well as in mammary arteries (Fig. 1). We found a predominant expression of RORα1 in hASMC, and RORα4 in EC, while no expression of RORα2 or RORα3 mRNA was detected. In normal arteries, RORα4 was predominant compared with RORα1. In human atherosclerotic plaques, RORα mRNA expression was significantly decreased (Fig. 2).

As revealed by RT-PCR analysis (Fig. 3, top), incubation of hASMC for 6 h with increasing concentration of IL-1β from 0 to 10 ng/ml, dose-dependently increased RORα mRNA expression. Quantification (Fig. 3, bottom) showed that the maximum increase in the RORα mRNA expression was obtained at 10 ng/ml of IL-1β ($150 \pm 40\%$, $P < 0.05$).

Since RORα is a transcriptional regulator, we studied its expression kinetics to determine whether it was likely to act on early or late genes. We stimulated hASMC with IL-1β

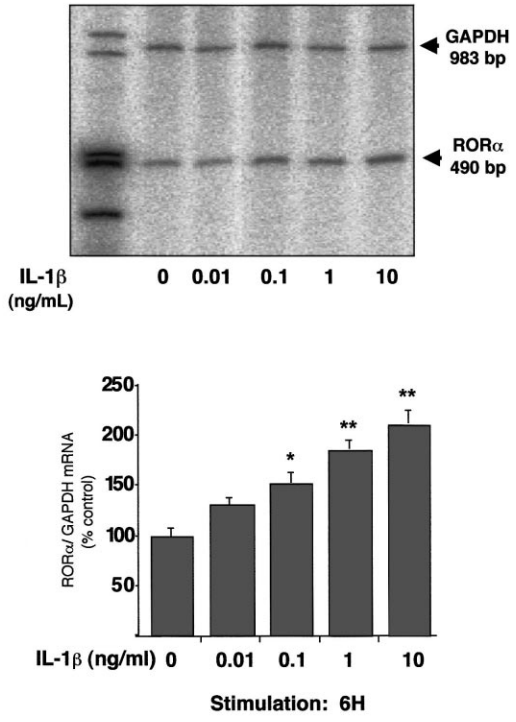

Fig. 3. Top: Representative RT-PCR of RORα and GAPDH under stimulation by increased doses of IL-1β. Bottom: Quantification of RT-PCR. Results shown are mean ± S.E.M. ($n = 4$).

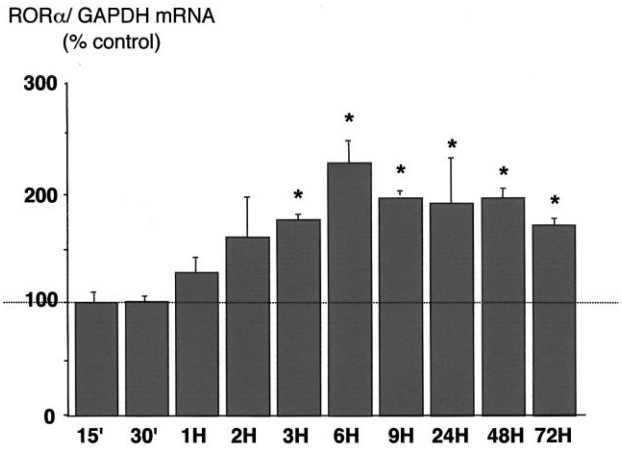

Fig. 4. Cells were incubated with the inflammatory cocktail IL-1β (10 ng/ml), TNFα (10 U/ml) and LPS (10 μg/ml) for the indicated times. 50 ng of total RNA was assayed for 25 and 26 cycles for RORα and GAPDH, respectively. Amplified products are electrophoresed onto a 5% polyacrylamide gel.

(10 ng/ml) for 15 min to 72 h (Fig. 4). RORα mRNA expression increased significantly at 2 h, and reached a maximal level at 6 h ($160 \pm 53\%$ of control). RORα mRNA expression remained elevated between 6 and 72 h. In order to evaluate whether RORα mRNA could be regulated by other cytokines than IL-1β, we investigated the effects of TNFα, LPS and IL-1β separately or in conjunction (Fig. 5). Each cytokine induced a significant increase (1.5- to 2.5-fold) in RORα mRNA expression, the most potent activation being by IL-1β. To evaluate whether RORα was also up-regulated by cytokines in EC, we incubated EC with IL-1β (10 ng/ml) for 6 h. We found a similar increase in RORα mRNA expression compared with that observed in hASMC (data not shown).

Finally, to evaluate whether RORα can be stimulated by hypoxia, hASMC and EC were submitted to hypoxia for 3–6 h. We found that hypoxia induced a rapid and transient increase in RORα mRNA expression. RORα mRNA expression was increased 3 h after hypoxia ($205 \pm 18\%$ in SMC and $200 \pm 12\%$ in EC), and returned to baseline values after 6 h (Fig. 6).

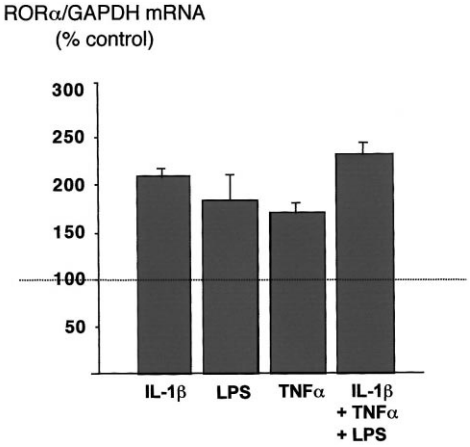

Fig. 5. Regulation of RORα mRNA expression by inflammatory cytokines in hSMC. hASMC (80% confluence) were incubated for 6 h in SmBM medium with IL-1β (10 ng/ml), TNFα (10 U/ml) and LPS (10 μg/ml), used alone or within a cocktail. RORα and GAPDH mRNA levels were measured by RT-PCR. Results shown are mean ± S.E.M. ($n = 5$).

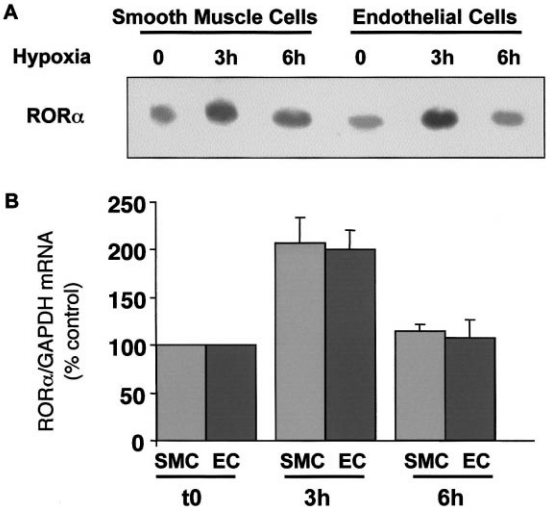

Fig. 6. A: Representative RT-PCR of RORα mRNA expression under hypoxic conditions (95% N_2, 5% O_2). B: Quantification of RT-PCR.

4. Discussion

In the present study, we showed that RORα, a member of orphan nuclear-receptor family, is expressed in vascular cells as well as in human arteries. We report for the first time a predominant expression of RORα1 in hASMC, and RORα4 in EC, while no expression of RORα2 or RORα3 mRNA was detected. In normal arteries, RORα4 was predominant compared with RORα1. However, RORα mRNA expression was significantly decreased in human atherosclerotic plaques. In both EC and hASMC, inflammatory cytokines IL-1β and TNFα, as well as LPS, increased by about two-fold RORα mRNA expression. Analysis of the RORα mRNA expression in response to IL-1β reveals that the time course of the activation was rapid (≈200% after 2–3 h), and sustained (up to 72 h).

Nuclear receptors play an important role in the regulation of numerous cellular functions. They relieve the different physiological effects of their ligands by activating specific genetic programs. The major role of nuclear receptors is to modulate transcription. They have been especially studied in embryonic development and in cancer [10–12]. However, little is known on the expression of nuclear receptors in vascular cells. Recent studies reported that endothelial cell proliferation could be modulated by retinoid and steroid hormones via their nuclear receptors [13,14]. Another nuclear receptor, peroxisome proliferator activated receptor-α (PPARα), has been shown to be expressed in endothelial cells [15] and SMC [16]. Its activation by specific ligands inhibits the inflammatory response in these cells [15,16]. A second subtype of PPAR, PPARγ, might also be expressed in human vascular SMC and its activation inhibits migration [17].

Several studies have shown that nuclear receptors might regulate cytokines production. PPAR nuclear-receptor family has been shown to modulate TNF-α, IL-1β and IL-6 expression by monocytes [18]. Retinoic acid and vitamin D3 receptors regulate at a transcriptional level the expression of inflammatory cytokine genes [19,20]. However, the effect of proinflammatory cytokines on nuclear receptor expression has been poorly studied. In a previous study, we showed abnormalities of IL-1β production in peripheral immune system of *staggerer* mice, which carry a deletion in the RORα gene. In these mutants, LPS-activated peritoneal macrophages overexpress both IL-1β mRNA and protein [21]. Other members of the RZR/ROR family, such as RORγt, seem to be involved in the modulation of cytokine expression: RORγt down-regulates IL-2 production [22].

In the present work, we found that RORα mRNA expression was enhanced after only 2 h of activation by cytokines, and remained elevated up to 72 h. This time course of RORα mRNA expression in the presence of cytokines might suggest the existence of regulation loops of cytokine production via RORα. However, in the absence of RORα activator(s) or inhibitor(s), this hypothesis cannot be tested. Carlberg et al. proposed that the pineal gland hormone melatonin might be a natural ligand for RORα [23], but other groups did not confirm this finding. A thiazolidinedione derivative, CGP 52 608, has also be reported to be a synthetic ligand of RORα [23]. The development of RORα activator(s) or inhibitor(s) could allow us to take a long stride in trying to understand how this new therapeutic target, RORα, could work in atherosclerosis and other inflammatory diseases.

While RORα is constitutively expressed in normal vessels, it is decreased in some of the atherosclerotic plaques analyzed. Interestingly, the *staggerer* mice (with RORα deletion) exhibit exagerated atherosclerosis compared with wild type mice [7].

A RORα-responsive element has been identified in the promoter of the 5-lipoxygenase gene, which may mediate the negative regulation of this important inflammatory gene [24]. Furthermore, RORα might play a major role in chronic inflammation. Peripheral macrophages from sg/sg mice produce greater amounts of proinflammatory cytokines, IL-1 and IL-6, under LPS stimulation [25]. Moreover, RORα1 has recently been reported to interfere negatively with inflammatory pathways in smooth muscle cells [9].

There is a striking correlation between inflammatory gene induction and susceptibility to fatty streak development in several inbred mouse strains. As we previously reported, RORα up-regulates apolipoprotein A-I expression, which elevated plasmatic levels confer protection against atherosclerosis [7].

Furthermore, we found a rapid, but transient induction of RORα mRNA expression by hypoxia in hASMC and EC. This result gives another trail for a role of RORα in the vascular physiology and pathology, and is in agreement with our recent finding that RORα expression is increased in the mouse ischemic hindlimb following femoral artery ligature [8]. Therefore, RORα might play a role in the control of ischemia-induced angiogenesis, as suggested by increased angiogenesis in sg/sg mice [8].

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