Increased Ischemia-Induced Angiogenesis in the Staggerer Mouse, a Mutant of the Nuclear Receptor Rorα

Sandrine Besnard, Jean-Sébastien Silvestre, Micheline Duriez, Joëlle Bakouche, Yolande Lemaigre-Dubreuil, Jean Mariani, Bernard I. Levy, Alain Tedgui

Abstract—Rora is an orphan nuclear receptor. In homozygous staggerer mutant mice (Rora<sup>sg/sg</sup>), a deletion within the Rora gene leads to an overexpression of inflammatory cytokines. Because inflammation and hypoxia are 2 key stimuli of ischemia-induced angiogenesis, we studied the role of Rora in this setting. Ischemia was induced by ligation of the right femoral artery in C57BL/6 Rora<sup>+/+</sup> and Rora<sup>sg/sg</sup> mice. After 3 and 28 days, angiogenesis was evaluated by microangiography, measurement of capillary density using immunohistochemistry (anti-CD31), and measurement of blood flow by laser Doppler imaging. At day 3, angiographic score and blood flow were similar in Rora<sup>sg/sg</sup> mice and in Rora<sup>+/+</sup> littermates. Conversely, at day 28, Rora<sup>sg/sg</sup> mice showed a significant 2-fold increase in angiographic score and a 3-fold increase in capillary density within the ischemic hindlimb compared with control. Functionally, this coincided with a significant rise in leg perfusion in Rora<sup>sg/sg</sup> mice (0.83 ± 0.05 for ischemic/nonischemic leg perfusion ratio) compared with Rora<sup>+/+</sup> mice (0.66 ± 0.04, P < 0.05). In addition, more extensive angiogenesis in Rora<sup>sg/sg</sup> mice correlated with an increased expression of eNOS protein by 83 ± 12% and 71 ± 24% at 3 and 28 days, respectively (P < 0.05), whereas the level of the antiangiogenic cytokine IL-12 was significantly reduced by 38 ± 10% at day 28 (P < 0.05). Conversely, no changes in VEGF expression were observed. Our study identifies for the first time a new role for Rora as a potent negative regulator of ischemia-induced angiogenesis. (Circ Res. 2001;89:1209-1215.)

Key Words: Rora m nuclear receptors m angiogenesis m ischemia m inflammation

Nuclear receptors are increasingly recognized as playing a major role in vascular biology. Peroxisome proliferator-activated receptors (PPARs) have received much attention over the last years on account of their antiinflammatory activities in vascular cells.1 More recently, activation of the retinoid X receptor (RXR) has been reported to decrease atherosclerosis in apoE knockout mice.2 We have previously shown that the absence of retinoic acid receptor-related orphan receptor (Ror) α is responsible for a hypoalphalipoproteinemia and is implicated in the development of atherosclerosis.3 Rora belongs to the nuclear receptor superfamily for which no natural ligand has yet been identified.4 The distribution of Rora mRNA suggests that this nuclear receptor is widely expressed and functions in several organs including the brain, heart, liver, testis, skin, and bone.5-7 A spontaneous mutation consisting in a 122-bp deletion, which removes the start of the ligand binding domain, shifts the reading frame, and causes a stop codon after 27 amino acids, has been identified in the staggerer mice.8 The homozygous staggerer (Rora<sup>sg/sg</sup>) mutant mouse is characterized by significant cerebellar abnormalities,9,10 defects in bone formation and maintenance,7 and a marked development of atherosclerotic lesions in response to an atherogenic diet.3 Moreover, peripheral macrophages from Rora<sup>sg/sg</sup> mice produce greater amounts of proinflammatory cytokines, IL-1 and IL-6, under lipopolysaccharide (LPS) stimulation.11 Interestingly, Rorα has recently been reported to interfere negatively with inflammatory pathways in smooth muscle cells.12

The potential implication of Rora in various inflammatory processes prompted us to study its role in ischemia-induced angiogenesis. Angiogenesis designates the process whereby new capillary blood vessels are formed from preexisting ones. This tightly controlled process is crucial during development, but it is also involved in the pathogenesis of numerous diseases such as diabetic retinopathy, tumor growth, and ischemic diseases.13-17 In ischemic diseases, both hypoxia and inflammation are considered to represent fundamental stimuli for angiogenesis.18 The inflammatory response leads to the secretion of proinflammatory cytokines associated with the recruitment of monocytes/macrophages within the ischemic tissue.19,20

In this report, we assessed the role of Rora in ischemia-induced angiogenesis by studying Rora<sup>sg/sg</sup> mice with operatively induced hindlimb ischemia. We show that Rora mRNA expression is significantly increased in ischemic tissues of Rora<sup>sg/sg</sup> mice and that angiogenesis is markedly enhanced in Rora<sup>sg/sg</sup> mice. Furthermore, endothelial NO synthase (eNOS) protein content is increased in ischemic tissues of Rora<sup>sg/sg</sup> mice and that angiogenesis is marked...
mice, whereas the expression of the antiangiogenic cytokine IL-12 is decreased. Conversely, no changes in vascular endothelial growth factor (VEGF) expression were observed. We conclude that Rorα functions as a negative regulator of ischemia-induced angiogenesis.

**Materials and Methods**

**Animals**
The Rora<sup>−/−</sup> mutant mice used in this study were maintained on a C57BL/6J genetic background in our colony. Rora<sup>−/−</sup> mutant mice and their Rora<sup>+/−</sup> littermates were obtained by crossing known heterozygous (Rora<sup>+/−</sup>) mice. Homozygous Rora<sup>−/−</sup> offspring were identified by their clinical ataxia, whereas Rora<sup>+/+</sup> and Rora<sup>−/−</sup> mice were genotyped by polymerase chain reaction (PCR). All mice were maintained in a temperature-controlled room (25°C) with a 12-hour light-dark cycle. The animals received a standard diet (A04, UAR, Epinay sur Orge, France) and water ad libitum.

**Genotype Analysis**
The homozygous Rora<sup>−/−</sup> mice were detected on the basis of their phenotype. The other animals were genotyped by PCR. Genomic DNA was extracted from tail biopsies and amplified in 2 sets of reaction, one for each allele. The staggerer allele primers were: 5′-CGTTGGCAAACTCACC-3′ and 5′-GATTGAAAGCTGACTCGTTCC-3′. The + allele primers were: 5′-CTCTCCCTTCAGTCTGAC-3′ and 5′-TATATTCCACCAACGGCAA-3′. The amplified fragments (318 bp + and 450 bp sg) were detected by electrophoresis on agarose gel.

**Experimental Protocol**
Male C57BL/6 Rora<sup>−/−</sup> mice and Rora<sup>+/−</sup> mice underwent surgery to induce unilateral hindlimb ischemia as previously described. Briefly, animals were anesthetized by isoflurane inhalation. The ligature was performed on the right femoral artery and to inject a contrast medium (Barium sulfate, 1 g/mL). Angiography of the hindlimbs was then assessed and aorta and to inject a contrast medium (Barium sulfate, 1 g/mL). Angiography of the hindlimbs was then assessed and 7 per group) were then processed in Rora<sup>−/−</sup> and Rora<sup>+/−</sup> mice (n=7 per group) were then housed under standard conditions for 3 or 28 days.

**Microangiography**
Vessel density was evaluated by high-definition microangiography at the end of the 3 or 28 days treatment period, as previously described. Mice were anesthetized (isoflurane inhalation) and a longitudinal laparotomy was performed in order to introduce a polyethylene catheter into the abdominal aorta and to inject a contrast medium (Barium sulfate, 1 g/mL). Angiography of the hindlimbs was then assessed and images (3 per animal) were acquired by a digital X-ray transducer. Images were then assembled in order to obtain a complete view of the hindlimbs. The angiographic score was expressed as a percentage of pixels per image occupied by vessels in the quantification area. The quantification zone was delimited by the point of ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg.

After microangiography analysis, the back muscle of thigh was dissected both in ischemic and nonischemic hindlimb, to perform histological, Western blot, and reverse transcriptase (RT)-PCR studies.

**Determination of Rora mRNA Expression**

**Total RNA Isolation**
Total RNA was extracted from tissues, according to the Trizol reagent protocol (Life Technologies). The quality or RNA was confirmed by ethidium bromide staining in 1% agarose gel.

**RT-PCR Protocol**
Rora mRNA was amplified using primers specific of the common part of the 4 isoforms of Rora. First strand cDNA was performed on 50 ng of total RNA. PCR conditions were as follows: 39°C, 60 minutes; 94°C, 3 minutes; 94°C, 45 seconds; 55°C, 1 minute; and 72°C, 1 minute. The PCR fragments were visualized on a 5% polyacrylamide gel. Primers and annealing temperatures used were as follows: Rora, forward primer 5′-GTCAGCAGTCCTCTACCTGGAC-3′ and reverse primer 5′-TGTTGGTCTGTGAGGTGAAG-G-GACG-3′, 5S; GAPDH forward primer 5′-TGAAGGTCTGGTCAACCGAGATTTGGC-3′ and reverse primer 5′-GTGGTGGTCTGTGAGGTGAAG-G-GACG-3′, 5S.

**Capillary and Arteriole Densities**
Microangiographic analysis was completed by assessment of capillary and arteriole density. Ischemic and nonischemic muscles were dissected and progressively frozen in isopentane solution cooled in liquid nitrogen. After fixation for 10 minutes in 50% acetone/50% methanol, sections (7 μm) were incubated for 5 minutes in 3% H<sub>2</sub>O<sub>2</sub> at room temperature and then for 1 hour with a rat monoclonal antibody directed against mouse CD-31 (Pharmingen, 20 μg/mL) to identify endothelial cells or with an alkaline phosphatase-conjugated mouse monoclonal antibody directed against smooth muscle α-actin (Sigma, dilution 1:20) to identify arterioles. Capillaries were revealed with NBT/BCIP substrate of alkaline phosphatase (Boehringer). Capillary and arteriole densities were then calculated in a 7 to 10 randomly chosen fields per muscle using Optilab Pro software. In the same time, muscle fiber density was calculated in randomly chosen fields.

**Laser Doppler Perfusion Imaging**
To provide functional evidence for ischemia-induced changes in local blood flow, laser Doppler perfusion imaging experiments were performed in Rora<sup>−/−</sup> and Rora<sup>+/−</sup> mice (n=7 in each group), as previously described.

**Determination of Protein Expression**
Total proteins were extracted from frozen tissues as previously described. Protein content was then determined by the method of Bradford. The same amount of each sample was then loaded in denaturing SDS/9% polyacrylamide gels. Polyclonal antibodies (Santa Cruz) against the following proteins were used: VEGF (dilution 1:2000), eNOS (dilution 1:300), and IL-12 (dilution 1:1000). Specific protein was detected by chemiluminescent reaction (ECL+ kit, Amerham), and chemiluminescent signals were visualized using a computer-based imaging system (Fuji LAS 1000 plus, Fuji Medical system). As a protein loading control, membranes were stripped, incubated with a goat polyclonal antibody directed against total actin (Santa Cruz, dilution 1:100), and specific chemiluminescent signal was detected as described above. The proteins were then stained with Ponceau Red (Sigma) for 10 minutes. Quantifications were performed by densitometric analysis after scanning using the Bio-Rad gel Doc 1000. Results are expressed as a ratio of quantification of...
the specific band to quantification of the transferred total protein bands stained with Ponceau Red.

Data Analysis
Results are expressed as mean±SEM. Comparisons (t test) were performed to compare each parameter that was expressed as an ischemic/nonischemic leg ratio. A value of P<0.05 was considered statistically significant.

Results
Expression of Rora in Ischemic Hindlimb of Rora<sup>+/+</sup> Mice
We first verified whether Rora was expressed in the mouse hindlimb and whether ischemia could modify its expression. Ischemia induced an early activation of Rora mRNA expression, which was increased by 1.8-fold versus baseline level (ie, ischemic/nonischemic ratio=1), 3 hours after ligature (P<0.05), and returned to baseline 4 hours after ligature (Figure 1).

Microangiography
Three days after ligature, vessel density decreased by 50% in the ischemic (right) leg of both Rora<sup>+/+</sup> and Rora<sup>gh/gh</sup> mice compared with the nonischemic (left) one. However, ischemic/nonischemic angiographic score ratio was similar in Rora<sup>gh/gh</sup> and Rora<sup>+/+</sup> mice. In contrast, after 28 days, Rora<sup>gh/gh</sup> mice displayed a significant increase in angiogenesis, characterized by an 80±22% rise in vascular density in the ischemic hindlimb of Rora<sup>gh/gh</sup> compared with that of Rora<sup>+/+</sup> mice (ischemic/nonischemic vessel density ratio: 1.19±0.07 versus 0.59±0.06, respectively, P<0.001) (Figure 2). In the nonischemic hindlimb, the angiogenic score remained unchanged in both Rora<sup>gh/gh</sup> and Rora<sup>+/+</sup> mice (data not shown).

Capillary and Arteriole Densities
Microangiographic data obtained at day 28 were confirmed by capillary and arteriole density evaluation. Capillary density was 3-fold increased in the ischemic hindlimb of Rora<sup>gh/gh</sup> mice compared with Rora<sup>+/+</sup>. Ischemic/nonischemic capillary density ratios were 1.71±0.20 and 0.56±0.10 in Rora<sup>gh/gh</sup> and Rora<sup>+/+</sup> mice, respectively (P<0.01) (Figure 3). No differences in arteriole density were observed between Rora<sup>gh/gh</sup> and Rora<sup>+/+</sup> mice (ischemic/nonischemic arteriole density ratios: 1.01±0.07 and 0.97±0.07, respectively). Furthermore, muscle density was not affected in Rora<sup>gh/gh</sup> compared with Rora<sup>+/+</sup> mice (ischemic/nonischemic muscle fibers density ratios: 0.99±0.05 versus 1.01±0.02, respectively).

In the nonischemic hindlimb, capillary, arteriole, and muscle fibers densities remained unchanged in both Rora<sup>gh/gh</sup> and Rora<sup>+/+</sup> mice.

Laser Doppler Perfusion Imaging
At day 3, hindlimb ligation resulted in an acute reduction in right leg blood flow, the degree of which did not differ between Rora<sup>gh/gh</sup> and Rora<sup>+/+</sup> mice. However, the increase in both angiography and capillary density observed after 28
days in Rora^+/+ mice corresponded with a significant increase in leg perfusion. Indeed, at this time point, blood flow recovery was 1.2-fold increased in Rora^sg/sg mice compared with controls (ischemic/nonischemic leg perfusion ratios: 0.83±0.05 in Rora^+/+ mice versus 0.66±0.04 in Rora^+/+ mice, *P*<0.001) (Figure 4).

**Protein Expression**

To assess some of the molecular mechanisms associated with the angiogenic process, we measured protein levels of VEGF, eNOS, and IL-12 by Western blot analysis. No differences in VEGF expression were observed between Rora^sg/sg and Rora^+/+ mice, whatever the time after femoral ligation (Figure 5). However, more extensive angiogenesis in Rora^sg/sg mice correlated with an increased expression of eNOS in these animals at both 3 and 28 days (ischemic/nonischemic leg perfusion ratios: 1.83±0.12 in Rora^sg/sg mice versus 1.03±0.17 in Rora^+/+ mice, *P*<0.01 at 3 days, and 1.71±0.24 versus 0.75±0.18, *P*<0.05 at 28 days) (Figure 6). Interestingly, the levels of the antiangiogenic cytokine IL-12 were reduced at 28 days in Rora^sg/sg mice (ischemic/nonischemic leg ratios: 0.75±0.11 in Rora^sg/sg mice versus 1.13±0.14 in Rora^+/+ mice, *P*<0.05) (Figure 7).

**Discussion**

The present study identifies for the first time a new role for the orphan nuclear receptor Rorα as an important negative modulator of angiogenesis. We show that absence of a functional Rorα in Rora^sg/sg mice favors a more extensive revascularization of the mouse hindlimb. Our findings add to the current knowledge on the role of nuclear receptors as potential modulators of the angiogenic process. Hence, ligands of PPARγ have been reported to decrease mRNA levels of VEGF receptors, Flt-1 and Flk/kDR, leading to a reduction of revascularization.24 In contrast, activation of RXR stimulates the formation of endothelial capillary-like tubular structures.25

We found that Rora mRNA expression was rapidly and transiently induced by ischemia following femoral artery ligation. Although further studies will be needed to determine the precise mechanism by which this occurs, hypoxia and inflammation are likely candidates to account for this finding.18 Indeed, both hypoxia and release of inflammatory mediators characterize the onset of ischemia. Interestingly, Rora gene expression has recently been shown to be activated by the proinflammatory cytokine IL-1β in chondrocytes.26 However, given the rapidity with which Rora was activated in our model, at 3 hours after femoral ligation, it is most likely that hypoxia, rather than inflammation, was responsible for increased Rora gene expression in the ischemic hindlimb of Rora^+/+ mice. To what extent the rapid and transient 2-fold increase in Rora mRNA expression is involved in the marked...
Because vessel density does not differ between Rora<sup>sg/sg</sup> and Rora<sup>H9251</sup> limb of Rorasg/sg mice compared with controls, suggesting that expression was significantly enhanced in the ischemic hindlimb of Rorasg/sg mice, rather than the transient overexpression at 3 hours in Rora<sup>H9251</sup> mice, of transcription, it is likely that the lack of Rora mRNA accounts for our findings. Because vessel density does not differ between Rora<sup>sg/sg</sup> and Rora<sup>+/+</sup> mice in nonischemic tissue, our results point to a role for Rora in modulating the expression of ischemia-induced genes.

The cascade of events induced by ischemia and leading to revascularization is under the control of several transcription factors, including hypoxia-inducible factor-1 (HIF-1α) which presents an early peak of expression within the first hour of hypoxia. HIF-1α is known to in turn activate the proangiogenic factors VEGF and eNOS. VEGF exerts a pivotal role in normal and pathological angiogenesis and early synthesis of VEGF controls the onset, extent, and duration of vessel growth. However, in our study VEGF protein content in the ischemic leg did not differ between Rora<sup>sg/sg</sup> and Rora<sup>+/+</sup> mice, whatever the time point after femoral ligation, suggesting that Rora has no direct, or indirect, influence on VEGF expression. In contrast, eNOS expression was significantly enhanced in the ischemic hindlimb of Rora<sup>sg/sg</sup> mice compared with controls, suggesting that Rora negatively modulates eNOS protein content.

Hitherto, only positively regulated Rora target genes have been identified. Rora binds selectively as a monomer to the Rora response element (RORE) within the promoter region of target genes, composed of the consensus half-site of AGGTCA. In searching for genes whose transcription is activated by Rora, RORE sites have been identified in many gene promoters, including 5-lipoxygenase, CRABP-I (cellular retinoic acid-binding protein I), inhibitor of cyclin-dependent kinases, and bone sialoprotein gene. Moreover, Rora is able to directly induce myogenesis via the upregulation of MyoD and p300 expression. Therefore, our finding of enhanced eNOS expression in Rora<sup>sg/sg</sup> mice most likely indicates that Rora controls the expression of some unknown proteins that negatively interfere with eNOS expression. NO has been reported to be an effector of angiogenesis downstream from VEGF. Spontaneous angiogenesis occurring after surgically induced hindlimb ischemia is severely impaired in eNOS knockout mice. We therefore believe that elevated eNOS expression might account for the more extensive revascularization observed in the ischemic hindlimb of Rora<sup>sg/sg</sup> mice.

The angiogenic process is also controlled by inflammation. Interestingly, Rora1 has recently been shown to interfere negatively with the nuclear factor (NF)-κB signaling pathway by reducing p65/NF-κB translocation. LPS-activated peritoneal macrophages from Rora<sup>H9251</sup> mice overexpress IL-1β. Therefore, the exaggerated inflammatory profile of Rora<sup>sg/sg</sup> mice might account for the observed increased ischemia-induced angiogenesis. The angiogenic reaction is also modulated by the balance between proangiogenic and antiangiogenic factors. Among the latter, IL-12 is of great interest for it is a proinflammatory cytokine secreted by...
macrophages, which potentially inhibits angiogenesis.35,36 We observed a significant decrease in IL-12 protein levels in Rora<sup>−/−</sup> mice compared with controls, suggesting that Rorα upregulates IL-12 expression. This identifies another pathway through which Rorα may act to hinder vessel growth.

Taken together, the results obtained in in vivo studies implicate an important role for Rorα in angiogenesis and show that Rora mRNA expression is rapidly and transiently increased by ischemia. Further studies will be needed to determine the exact mechanisms of ischemia-induced Rora activation. The downstream events that might account for the antiangiogenic effect of Rora remain to be fully elucidated. However, we clearly identified 2 pathways associated with the antiangiogenic function of Rora, one leading to the downregulation of eNOS and the other resulting in the upregulation of IL-12.

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**References**


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