Retinoid X Receptor Agonists Impair Arterial Mononuclear Cell Recruitment through Peroxisome Proliferator-Activated Receptor-\(\gamma\) Activation

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Retinoid X Receptor Agonists Impair Arterial Mononuclear Cell Recruitment through Peroxisome Proliferator-Activated Receptor-\(\gamma\) Activation

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Mononuclear cell migration into the vascular subendothelium constitutes an early event of the atherogenic process. Because the effect of retinoid X receptor (RXR) on arterial mononuclear leukocyte recruitment is poorly understood, this study investigated whether RXR agonists can affect this response and the underlying mechanisms involved. Decreased RXR expression was detected after 4 h stimulation of human umbilical arterial endothelial cells with TNF-\(\alpha\). Interestingly, under physiological flow conditions, TNF-\(\alpha\)-induced endothelial adhesion of human mononuclear cells was concentration-dependently inhibited by pre-incubation of the human umbilical arterial endothelial cells with RXR agonists such as bexarotene or 9-cis-retinoic acid. RXR agonists also prevented TNF-\(\alpha\)-induced VCAM-1 and ICAM-1 expression, as well as endothelial growth-related oncone-\(\alpha\) and MCP-1 release. Suppression of RXR expression with a small interfering RNA abrogated these responses. Furthermore, inhibition of MAPKs and NF-\(\kappa\)B pathways were involved in these events. RXR agonist did not affect leukocyte adhesive effects seemed to be mediated via RXR-\(\alpha\)/peroxisome proliferator-activated receptor (PPAR)\(\gamma\) interaction, since endothelial PPAR\(\gamma\) silencing abolished their inhibitory responses. Furthermore, RXR agonists increased RXR/PPAR\(\gamma\) interaction, and combinations of suboptimal concentrations of both nuclear receptor ligands inhibited TNF-\(\alpha\)-induced mononuclear leukocyte arrest by 60–65%. In vivo, bexarotene dose-dependently inhibited TNF-\(\alpha\)-induced leukocyte adhesion to the murine cremasteric arterioles and decreased VCAM-1 and ICAM-1 expression. Therefore, these results reveal that RXR agonists can inhibit the initial inflammatory response that precedes the atherogenic process by targeting different steps of the mononuclear recruitment cascade. Thus, RXR agonists may constitute a new therapeutic tool in the control of the inflammatory process associated with cardiovascular disease. The Journal of Immunology, 2012, 189: 000–000.

Atherosclerosis bears several histopathologic similarities to chronic inflammation. The early atherosclerotic lesion involves an inflammatory response consisting of the intimal accumulation of T lymphocytes and lipid-laden macrophages, which occurs throughout the entire atherogenic process (1, 2). One of the earliest stages of atherogenesis is endothelial dysfunction. Endothelial dysfunction leads to a proinflammatory and prothrombotic phenotype of the endothelium that provokes the attachment and subsequent migration of leukocytes (3). Leukocyte/arterial interactions require both the expression of various types of cell adhesion molecules (CAMs) in the endothelium and leukocytes, as well as the presence of counterreceptor molecules in the leukocyte/endothelial cell (1, 2).

In addition to adhesion molecules, chemoattractant molecules, such as chemotactic cytokines or chemokines, have the potential to recruit specific cell types and are involved in the regulation of leukocyte trafficking (4). Chemokines are small, structurally related disulfide-linked polypeptides that are potent mediators of cell adhesion and migration through their interactions with a family of G protein-coupled receptors expressed on leukocytes. Several CC chemokines, including MCP-1/CCL2, seem to be involved in atherosclerotic lesion formation (4). To a lesser extent, CXC chemokines also appear to be involved in triggering the firm arrest of monocytes on the activated endothelium. Among them, growth-related oncogene (GRO)\(\alpha\) plays a pivotal role in mononuclear cell adhesion and in many inflammatory conditions, including atherosclerosis (4–6).

The process of endothelial dysfunction underlies tight regulatory control mechanisms. Proinflammatory stimuli targeting the endothelium such as TNF-\(\alpha\) elicit the activation of intracellular signal transduction pathways. One of the most important is the activation of members of MAPK family: the ERKs, the p38 MAPKs, and JNKs (7). A growing interest has focused on the activation of MAPKs during endothelial dysfunction and its role in the pathogenesis of vascular diseases (8). Additionally, TNF-\(\alpha\) activation of MAPK signaling cascades result in NF-\(\kappa\)B transactivation (9), which leads to the expression of genes encoding different adhesion molecules and chemokines (10).
Retinoid X receptor (RXR)α is a member of the nuclear hormone receptor superfamily that mediates the biological effects of several hormones, vitamins, and drugs. RXRs act as a transcription factor that, on activation, binds to gene regulatory DNA sequences and subsequently mediates the transcription of target genes (11). Moreover, RXRs also dimerize with other nuclear hormone receptors such as the farnesoid X receptor (also known as the bile acid receptor), the liver X receptors, and the peroxisome proliferator-activated receptors (PPARs), thereby affecting numerous signal transduction pathways associated with the regulation of glucose and lipid metabolism (12). Additionally, 9-cis-retinoic acid (9-cis-RA) was originally identified as a natural ligand of RXRs that was first detected in mouse kidney, liver, and more recently in pancreatic β cells as well as in human spermatozoa (13-15). In contrast, bexarotene is a synthesized drug and an RXR-selective retinoid ligand that did not activate retinoid acid receptor (RAR)-dependent genes and is less toxic than naturally occurring retinoids or RAR-selective retinoids (16). It is used in the treatment of cutaneous T cell lymphoma (16).

At present, there is enormous interest in dissecting the counterregulatory molecular mechanism activated by endogenous mediators to bring about inflammatory resolution and restoration of tissue homeostasis (17). In this context, RXR ligands have been shown to suppress different steps of the inflammatory process in several in vivo and in vitro models of inflammation (11), to reduce the progression of atherosclerosis in apolipoprotein E knockout mice (18, 19), and to impair high glucose-induced oxidative stress in human endothelial cells (20).

To date, whether RXRs regulates one of the most critical aspects of the inflammatory responses, leukocyte recruitment, is unknown. The present study has shown that RXR activation inhibits TNF-α-induced leukocyte/endothelial cell interactions in vitro under laminar flow conditions and in vivo in the murine cremaster microcirculation. These anti-inflammatory effects correlate with the downregulation of CAM expression and inhibition of endothelial release of chemokines such as GROα or MCP-1. Finally, the study has also found that activation of PPARγ, but not PPARα or PPARβ, was involved in the arterial anti-inflammatory effects displayed by RXR agonists.

Materials and Methods

Human studies

All research with human samples in the present study complied with the principles outlined in the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the University Clinic Hospital of Valencia (Valencia, Spain). Written, informed consent was obtained from all volunteers.

Cell culture

Human umbilical arterial cells (HUAECs) were isolated by collagenase treatment (21) and maintained in human endothelial cell-specific medium (endothelial basal medium-2) supplemented with endothelial growth medium-2 and 10% FCS. Cells up to passage one were grown to confluence on (endothelial basal medium-2) supplemented with endothelial growth medium-2 and 10% FCS. Cells up to passage one were grown to confluence on human endothelial cell-specific medium (HUAECs up to passage one were grown to confluence and stimulated with medium containing 1% FCS and then returned to the 10% FCS medium at 24-well culture plates. Prior to every experiment, cells were incubated 16 h in medium containing 1% FCS and 10% FCS. Cells up to passage one were grown to confluence and stimulated with medium containing 1% FCS and then returned to the 10% FCS medium at 24-well culture plates. Prior to every experiment, cells were incubated 16 h.

Leukocyte/HUAEC interactions under flow conditions

HUAECs up to passage one were grown to confluence and stimulated with TNF-α (10 ng/ml) for 4 h. Bexarotene, 9-cis-RA, and/or a PPARα agonist (GW7647), a PPARβ agonist (GS501516), or a PPARγ agonist (rosiglitazone) were added to some plates 20 h prior to TNF-α stimulation. Human mononuclear cells were obtained from buffy coats of healthy donors by Ficoll-Hypaque density gradient centrifugation. The GlycoTech flow chamber was assembled and placed onto an inverted microscope stage, and freshly isolated human mononuclear cells (1 × 10⁶/ml) were then perfused across the endothelial monolayers. In all experiments, leukocyte interactions were determined after 5 min at 0.5 dynes/cm². Cells interacting on the surface of the endothelium were visualized and recorded (×20 objective, ×10 eyepiece) using phase-contrast microscopy.

Quantification of mRNA expression by real-time PCR

Total RNA was isolated from cultured HUAECs by using TRizol isolation reagent (Invitrogen, Carlsbad, CA). The reverse transcription was performed in 300 ng total RNA with TaqMan reverse transcription reagents kit (Applied Biosystems/PerkinElmer, Foster City, CA). The CDNA was amplified with specific primers RXRα, PPARα, PPARγ, ICAM-1, VCAM-1, and GAPDH (all predesigned by Applied Biosystems) in a 7900HT Fast real-time PCR system (Applied Biosystems) usingUniversal PCR Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the 2⁻ΔΔCT method using GAPDH as the endogenous control and normalized to control group.

RXRs silencing via small interfering RNA

Confuent HUAECs were transfected with either control or RXRs, PPARα, PPARγ, or PPARγ-specific small interfering RNA (siRNA) (Dharmacon, Lafayette, CO) using Lipofectamin RNAiMAX (Invitrogen, Carlsbad, CA) for 48 h, as previously described (22). The mRNA expression for transcripts was determined by real-time RT-PCR after 48 h postincubating and compared with the siRNA control at the respective time to determine silencing efficiency. Furthermore, protein expression was measured by Western blot after 48 h silencing. Thereafter, cells were treated for 20 h with bexarotene (1 μM) or 9-cis-RA (1 μM) or vehicle, and were then stimulated for 4 h with TNF-α (10 ng/ml).

Quantification of endothelial adhesion molecule expression

HUAEC expression of VCAM-1 and ICAM-1 was analyzed by RT-PCR (as described above) and image flow cytometry. In brief, HUAECs were gently detached with PBS containing 0.05% trypsin, fixed with 2% paraformaldehyde, and blocked with PBS containing 3% BSA for 15 min. Cells were then suspended in PBS containing 0.5% BSA and incubated for 30 min with a 1:100 dilution of primary Abs against VCAM-1 (clone 1.G11B1; Serotec, Kidlington, U.K.) or ICAM-1 (clone 6.5B5; Serotec, Kidlington, U.K.). Detection of primary Abs was performed using an appropriate Alexa Fluor 488 secondary Ab (Molecular Probes/Invitrogen, Carlsbad, CA; dilution 1:250). Cells were then analyzed using an Amnis ImageStream™ IS100 and the IDEAS image analysis software package (Amnis, Seattle, WA).

Determination of CD11b/CD18 expression on human monocytes

Monocyte expression of CD11b and CD18 integrin was determined in heparinized whole blood obtained from healthy donors. Whole blood samples (100 μl) were incubated for 20 h with bexarotene (0.1–30 μM) or 9-cis-RA (0.1–30 μM) or vehicle, and then stimulated for 4 h with TNF-α (10 ng/ml). Samples were then incubated for 20 min on ice in the dark with saturating amounts (10 μl) of the FITC-conjugated mAb against human CD11b/CD18 (clone ICRF 44; Serotec, Kidlington, U.K.). RBCs were lysed and leukocytes fixed using an automated EPICS Q-PREP system (Coulter Electronics, Hialeah, FL). Samples were run in the EPICS XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL).

EMSA

Nuclear extracts were prepared with the NE-PER nuclear extraction kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. The LightShift chemiluminescent EMSA kit (Pierce) was used for band detection since a nonisotopic method to detect DNA/protein interactions was employed. A biotin end-labeled DNA duplex of sequences 5’-AGTTCAGGG-GACTTTCCCGG-3’ and 3’-TCACAATCCCTGAAGGGTCC-5’ containing a putative binding site for NF-κB was incubated with the nuclear extracts. The binding reactions contained 5 μg nuclear extract protein, buffer (10 mM Tris [pH 7.5], 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.05% Nonidet P-40, and 2.5% glycerol), 1 μg poly(dI-dC), and 2 mM biotin-labeled DNA. The samples were incubated for 20 min at room temperature. The competition reaction was performed by adding 200-fold excess of unlabeled double-stranded consensus oligonucleotide for NF-κB to the reaction mixture. Additionally, a reaction was performed with a mutation sequence in the NF-κB motif. The reaction products were electrophoresed on a 6% precast Tris-borate-EDTA gel at 100 V for 1 h in a 100 mM Tris-borate-EDTA buffer and then transferred to a nylon membrane (Biodyne B membranes). The membrane was immediately cross-linked for 15 min on a UV transilluminator. A chemiluminescent detection method utilizing a luminal/enhancer solution and a stable peroxide solution was used as described by the manufacturer (Pierce).
Western immunoblotting and immunoprecipitation

Western analysis was performed as previously described (22). After treatment, cells were washed, scraped, collected, and centrifuged at 15,000 × g at 4°C for 30 min to yield the whole extract. Protein concentration was determined by the Bradford method. Samples were denatured, subjected to SDS-PAGE using a 10% running gel, and transferred to a nitrocellulose membrane. Membranes were blocked with 5% BSA-TBS containing 0.05% Tween 20, and then incubated with the corresponding Ab following the manufacturer’s recommendations. The rabbit polyclonal Abs against RXRα and PPARγ (H-100) were from Santa Cruz Biotechnology (Santa Cruz, CA; dilution: 1:500) and against PPARα and PPARβ were from Abcam (Cambridge, U.K.; dilution, 1:500). Primary Abs of NF-κB p65 (c-20), phospho-NF-κB p65 (Ser536), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p44/p42 MAPK/ERK1/2, phospho-p44/p42 MAPK/ERK1/2 (Thr202/Tyr204), stress-associated protein/JNK, and phospho–stress-associated protein kinase/JNK (Thr183/Tyr185) and the secondary HRP-linked anti-rabbit IgG Ab were supplied by Cell Signaling Technology (Danvers, MA), all diluted at 1:1,000.

For immunoprecipitation, cell extracts were prepared in 50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 1% Nonidet P-40, and protease (1 mM PMSF, 40 μg/ml aprotinin, and 40 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate and 1 mM Na3P04). Protein (~200 μg) from cell extracts was incubated with 5 μg Ab against PPARγ. Immunocomplexes were precipitated using anti-rabbit IgG beads (eBioscience, San Diego, CA), following the manufacturer’s instructions, and suspended in sample buffer containing fresh 50 mM DTT. Western blot was performed with an Ab against RXRα. Membranes were developed using HRP-conjugated rabbit TrueBlot (eBioscience, San Diego, CA) as secondary Ab. Chemiluminescent signals were developed with ECL (GE Healthcare, Madrid, Spain).

Immunofluorescence

Confluent endothelial cells were grown on glass coverslips. Some coverslips were treated for 20 h with bexarotene (1 μM) or 9-cis-RA (1 μM) or vehicle, and then stimulated for 4 h with TNF-α (10 ng/ml). The cells were then washed with PBS, fixed with 4% paraformaldehyde, and blocked in a 1% PBS/BSA solution. NF-κB localization was visualized using a TCS SP2 confocal microscopy (Leica, Wetzlar, Germany) in HUAEC monolayers by indirect immunofluorescence, according to a previously described protocol (23). A primary Ab against the NF-κB p65 subunit (Transduction Laboratories, Lexington, KY; dilution, 1:100) and an Alexa Fluor 488-conjugated secondary Ab (Molecular Probes/Invitrogen; dilution, 1:250) were used.

Chemokine detection

Human chemokines MCP-1 and GRO-α were measured in HUAEC culture supernatants using Ab pairs from R&D Systems (Abingdon, U.K.). After coating the plates overnight with the primary Ab, non-specific binding sites were blocked with 3% BSA for 1 h. Supernatants and standards were added to PBS/0.5% BSA/0.05% sodium azide for 2 h. Biotinylated detector Abs were added for 2 h, followed by neutravidin-HRP for 1 h. All plate washes were carried out in four cycles in freshly made PBS/0.2% Tween 20. Enhanced K-Blue tetramethylbenzidine substrate was added for 30 min and the enzyme reaction was stopped by the addition of 0.19 M sulfuric acid. Ab-sorbance was read at 450 nm and the data were processed by GraphPad Prism software. Results are expressed as picomolar chemokine in the supernatant.

Animal studies

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and was approved by the Ethics Review Board of the School of Medicine, University of Valencia. Mice (C57BL/6) were supplied by Charles River Laboratories (Barcelona, Spain). Animal colonies were bred and maintained under specific pathogen-free conditions. For the entire experimental period, the mice were fed with autoclaved balanced diet and water. The animals used were 22–30 g weight.

Intravital microscopy

Mice were anesthetized by i.p. injection with a mixture of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (200 mg/kg). The mouse cremaster preparation used in this study was similar to that described previously (24). The cremaster muscle was dissected from the tissues and exteriorized on an optical clear viewing pedestal. The muscle was cut longitudinally with a cautery and held flat against the pedestal by attaching silk sutures to the corners of the tissue. The muscle was then perfused continuously with warmed bicarbonate-buffered saline (pH 7.4) at a rate of 1 ml/min. An orthoscopic microscope (Nikon Optiphot-2, SMZ1) equipped with a video camera (Sony SSC-C530P) was used to capture images on videotape (Sony SVT-S3000P) for playback analysis. Cremaster venules or arterioles were selected for study and the diameter was measured online using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline blood cell velocity was also measured online using an optical Doppler velocimeter (Microcirculation Research Institute). Vessel blood flow was calculated from the product of mean RBC velocity (Vmean × centerline blood cell velocity/l.6) and cross-sectional area, assuming cylindrical geometry. Wall shear rate (γ) was calculated based on the Newtonian definition: γ = 8 × (Vmean/Dh)1/3, in which Dh is vessel diameter (25).

The number of rolling, adherent, and emigrated leukocytes was determined offline during playback of videotaped images. Rolling leukocytes were defined as those WBCs moving at a velocity less than that of erythrocytes in the same vessel. Leukocyte rolling velocity was determined from the time required for a leukocyte to move along 100 μm length of the microvessel and is expressed as micrometers per second. Flux of rolling leukocytes was measured as those cells that could be seen moving past a defined reference point in the vessel. The same reference point was used throughout the experiment because leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adhered. A leukocyte was defined as adherent, to arteriolar or venular endothelium, when it was stationary for at least 30 s. Leukocyte adhesion was expressed as the number per 100 μm length of vessel. Leukocyte emigration was expressed as the number of WBCs per microscopic field surrounding the vessel. In each animal, leukocyte responses were measured in three to five randomly selected arterioles or postcapillary venules.

Mice were treated orally by gavage with bexarotene (1, 10, 30 mg/kg) or with vehicle alone (carboxyethylcellulose 1%/polyethylene glycol 400/ Tween 20, 90/9.95/0.05) during a 20-h period. Then, the cremaster microcirculation was stimulated with 0.5 μg TNF-α in 0.3 ml isotonic saline by intracutural injection under anesthesia, and determinations were performed 4 h later. Doses of bexarotene were within the range previously used in rodents (26). At the end of the experiments, animals were humanely euthanized by anesthetic overdose.

Immunohistochemistry

For completion of the intravital microscopy measurements, the cremaster muscle was isolated and fixed in 4% paraformaldehyde, dehydrated using grade acetone washes at 4°C, and embedded in paraffin wax for localization of VCAM-1 and ICAM-1 as previously described (24). Tissue sections (4 μm thick) were incubated against mouse VCAM-1 or mouse ICAM-1 (1:50; BD Pharmingen, Franklin Lakes, NJ) overnight at 4°C, and then for a further 60 min at 37°C with a biotinylated anti-mouse secondary Ab (1:500 dilution, sc-2040; Santa Cruz Biotechnology, Santa Cruz, CA), streptavidin-HRP (Lab Vision, Fremont, CA), and diaminobenzidine substrate (Serotec, Düsseldorf, Germany). Slides were counterstained with hematoxylin. Positive staining was defined as an arteriole or venular endothelium, when it was stationary for at least 30 s. Leukocyte adhesion was expressed as the number per 100 μm length of vessel. Leukocyte emigration was expressed as the number of WBCs per microscopic field surrounding the vessel. In each animal, leukocyte responses were measured in three to five randomly selected arterioles or postcapillary venules.

Microcirculation was stimulated with 0.5 μg TNF-α in 0.3 ml isotonic saline by intracuteral injection under anesthesia, and determinations were performed 4 h later. Doses of bexarotene were within the range previously used in rodents (26). At the end of the experiments, animals were humanely euthanized by anesthetic overdose.

Statistical analysis

Differences between two groups were determined using a paired or unpaired Student t test, as appropriate. Differences between multiple groups were determined using a one-way analysis of variance (ANOVA) test, as appropriate. Differences between multiple groups were determined using Tukey’s honestly significant difference (HSD) test. Differences were considered statistically significant if p < 0.05.

FIGURE 1. TNF-α downregulates RXRα expression in human arterial endothelial cells. HUAECs were stimulated with TNF-α (10 ng/ml, 4 h). Quantification of RXRα protein expression was determined by Western blotting. Results are the means ± SEM of four independent assays. Graph represents values of densitometry of RXRα protein expression relative to β-actin. Representative gels are also shown. *p < 0.05 relative to values in the control group.
determined by ANOVA followed by a Student–Newman–Keuls multiple comparisons test when necessary. Values were expressed as means ± SEM. Data were considered statistically significant when $p$ was < 0.05.

Additional materials

Bexarotene was obtained from Axxora (BioVision, Mountain View, CA) and 9-cis-RA as well as GW7647 were obtained from Sigma-Aldrich (St. Louis, MO). Rosiglitazone and GW501516 were from Alexis Biochemicals (San Diego, CA). Natural and synthetic agonists were reconstituted in DMSO at a concentration of 10 mM. Recombinant murine and human TNF-α, GROα, MCP-1, and their respective Ab pairs for ELISA were obtained from R&D Systems (Minneapolis, MN). Neutavidin-HRP was supplied by Perbio Science (Cheshire, U.K.) and K-Blue substrate was supplied by Neogen (Lexington, KY). Unless stated, all other reagents were from Sigma-Aldrich.

Results

TNF-α causes downregulation of RXRα expression in HUAECs

TNF-α stimulation can affect RXRα expression in different cell types (27, 28). Therefore, we first investigated the effect of TNF-α on RXRα expression in primary cultures of HUAECs by Western blot. As illustrated in Fig. 1, stimulation of HUAECs with TNF-α (10 ng/ml) for 4 h significantly decreased constitutive endothelial RXRα expression.

RXRα activation inhibits TNF-α–induced leukocyte/endothelial cell interactions under flow conditions

Mononuclear leukocyte/endothelial cell interactions were evaluated in vitro using the dynamic flow chamber assay. For this purpose, freshly isolated human mononuclear cells were perfused across HUAEC monolayers stimulated or not with TNF-α (10 ng/ml) for 4 h. TNF-α caused a significant increase in mononuclear leukocyte rolling and adhesion to endothelial cells (Fig. 2), which was unaffected by the vehicle (0.01–0.03% DMSO; Fig. 2A, 2B). To determine the effects of RXR agonists on TNF-α–induced mononuclear cell recruitment, HUAECs were pretreated with either bexarotene or 9-cis-RA (0.1–3 μM) prior to TNF-α stimulation. Significant reductions in the mononuclear leukocyte rolling and adhesion produced by TNF-α were achieved in a concentration-dependent manner (Fig. 2). To confirm the observed effects, the next step was to silence the RXRα receptor in HUAECs. Endothelial cells were either transfected with control siRNA or RXRα

![FIGURE 2. RXR agonists inhibits TNF-α–induced mononuclear rolling and adhesion to HUAECs under physiological flow. Some cells were pretreated for 20 h with vehicle (DMSO, 0.01 and 0.03%), bexarotene (BEX), or 9-cis-RA (0.1–3 μM) prior to TNF-α stimulation (10 ng/ml, 4 h). Freshly isolated human mononuclear cells were perfused over the endothelial monolayers for 5 min at 0.5 dynes/cm² and leukocyte rolling (A, C, E) and adhesion (B, D, F) were quantified. Results are the means ± SEM of five to six independent experiments. *$p < 0.05$ relative to the vehicle group, †$p < 0.05$ relative to the TNF-α–stimulated cells.](http://www.jimmunol.org/)

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siRNA. Forty-eight hours after transfection with RXRα-specific siRNA, HUAECs showed a >75% reduction in RXRα mRNA compared with the levels detected in the control siRNA-transfected cells (Fig. 3A). Similarly, use of RXRα siRNA resulted in a >85% decrease in RXRα protein relative to that for control-transfected cells (Fig. 3B). Interestingly, RXRα-specific siRNA abolished the suppressive effects of RXR agonists on mononuclear leukocyte rolling (Fig. 3C) and adhesion (Fig. 3D) to the transfected HUAECs stimulated with TNF-α.

Effect of RXR agonists on endothelial cell and monocyte adhesion molecule expression

To investigate whether the inhibitory effects exerted by RXRα agonists on leukocyte rolling and adhesion were mediated through modulation of CAM expression, VCAM-1 and ICAM-1 mRNA and protein expression were determined by RT-PCR (Fig. 4A) and immunofluorescence (Fig. 4B), respectively. Stimulation with TNF-α resulted in a clear upregulation of both mRNA and protein levels of VCAM-1 and ICAM-1 when compared with unstimulated control HUAECs (Fig. 4A, 4B). Pretreatment of the cells with 1 μM bexarotene or 9-cis-RA resulted in decreased TNF-α–induced ICAM-1 and VCAM-1 expression (Fig. 4A, 4B). Interestingly, the suppressive effects of RXR ligands on TNF-α–induced ICAM-1 and VCAM-1 expression were absent in RXRα-deficient HUAECs (Fig. 4A, 4B).

Because CD11b/CD18 (also called α5β2 or Mac-1) expression is also involved in leukocyte adhesion to the arterial endothelium, the effect of RXRα ligands on this integrin was also investigated in human peripheral blood monocytes. TNF-α stimulation of whole blood for 4 h significantly increased CD11b/CD18 surface expression on human monocytes (Fig. 4C). Preincubation of the blood with 10 μM or 30 μM bexarotene or 9-cis-RA, however, did not modify TNF-α–induced CD11b/CD18 upregulation (Fig. 4C).

RXX agonists decrease TNF-α–induced MCP-1 and GROα release

Because TNF-α stimulation of endothelial cells can cause the release of different chemokines that contribute to mononuclear leukocyte arrest, we next evaluate the effect of RXR agonist in TNF-α–induced GROα and MCP-1 release from human arterial endothelial cells. Significant increases in the levels of GROα and MCP-1 were detected in the supernatant of HUAECs stimulated with TNF-α for 4 h. This effect was inhibited in a concentration-dependent manner by pretreatment of the cells with both RXR agonists (Fig. 5A, 5B). Conversely, when HUAECs were transfected with RXRα siRNA, neither bexarotene nor 9-cis-RA reduced chemokine release induced by TNF-α (Fig. 5C, 5D).

RXX agonists inhibit TNF-α–induced MAPK activation

To investigate the intracellular signaling pathways that underlie the inhibitory responses displayed by RXR agonists, HUAECs were stimulated with TNF-α for 30 min in the presence or absence of both RXR agonists. Bexarotene and 9-cis-RA at the dose of 1 μM dramatically decreased the TNF-α–induced phosphorylation of p38 MAPK and ERK1/2 (Fig. 6A, 6B). Although JNK phosphorylation by TNF-α was also significantly diminished, it was affected to a lesser extent than were the other two members of the MAPK family investigated (Fig. 6C). Nonphosphorylated p38 MAPK, ERK1/2, and JNK expression were not affected by TNF-α in the presence or absence of RXR agonists (Fig. 6).

Inhibition of TNF-α–induced NF-κB activation by RXR agonists

Given that MAPK signaling cascades precede TNF-α–induced NF-κB activation and that NF-κB signaling has a dominant role in mediating TNF-α–induced CAM expression and chemokine release (10), the following step was to investigate the effect of RXRα

FIGURE 3. siRNA knockdown of RXRα abolishes the inhibitory effect of RXR agonists on TNF-α–induced mononuclear leukocyte/endothelial cell interactions. Cells were transfected with RXRα siRNA or control siRNA. Knockdown of RXRα in HUAECs was determined by RT-PCR (A) and Western blotting (B). Results are the means ± SEM of four independent experiments. *p < 0.05 relative to the siRNA control group. Forty-eight hours after transfection cells were pretreated with bexarotene (BEX) or 9-cis-RA (1 μM, 20 h) and then stimulated with TNF-α (10 ng/ml, 4 h). Leukocyte rolling (C) and adhesion (D) were quantified. Results are the means ± SEM of four to five independent experiments. *p < 0.05 relative to the vehicle group, †p < 0.05 relative to the TNF-α–stimulated cells.
agonists on NF-κB activation. Immunofluorescence studies with confocal microscopy showed that both bexarotene (BEX) or 9-cis-RA undermined the nuclear translocation of NF-κB induced by TNF-α (Fig. 7A). Western blot analysis revealed that exposure of HUAECs to TNF-α produced increased p65 phosphorylation at Ser536, and phosphorylation was lowered when treating endothelial cells with RXRα agonists (Fig. 7B). Additionally, an EMSA assay also confirmed that bexarotene or 9-cis-RA HUAEC preincubation significantly reduced NF-κB binding activity induced by TNF-α (Fig. 7C).

PPARγ is involved in the anti-inflammatory response exerted by RXRα agonists

RXRα can dimerize with other nuclear hormone receptors such as the PPARs, and several studies have demonstrated that activation of PPARα, PPARβ, or PPARγ can modulate vascular inflammation (29). Therefore, we next investigated the potential involvement of PPARs in the inhibitory responses elicited by RXR agonists on mononuclear leukocyte arrest. Again, an siRNA approach was employed. As shown in Fig. 8, PPARα, PPARβ, or PPARγ was expressed in control siRNA arterial endothelial cells. Forty-eight hours after transfection with their respective siRNA, significant reductions in PPARα, PPARβ, and PPARγ mRNA and protein expression were achieved (Fig. 8). Notably, whereas PPARα or PPARβ siRNA-transfected cells did not reverse the inhibitory effects of bexarotene and 9-cis-RA in TNF-α–induced mononuclear cell rolling and adhesion, PPARγ knockdown did (Fig. 9).

To confirm the interaction between RXRα and PPARγ, HUAECs were initially immunoprecipitated with an anti-PPARγ Ab and
FIGURE 5. RXR activation inhibits GROα and MCP-1 release from TNF-α-stimulated HUAECs. GROα and MCP-1 release was determined by ELISA in the cell-free supernatant. HUAECs were treated with bexarotene (BEX) or 9-cis-RA (0.3–3 μM, 20 h) and then stimulated with TNF-α (10 ng/ml, 4 h) (A and B). Results are expressed as picomolar concentration and presented as means ± SEM of three to five independent experiments. *p < 0.05 relative to the TNF-α-stimulated group.

In another set of experiments endothelial cells were transfected with control or RXRα-specific siRNA. Twenty-four hours after transfection, cells were treated with RXR agonists for 20 h and then stimulated with TNF-α (10 ng/ml, 4 h). (C and D) Results are representative of four independent experiments. *p < 0.05 relative to the vehicle group. †p < 0.05 relative to TNF-α-stimulated cells.

then electrophoresed and Western blotted with an anti-RXRα Ab. As shown in Fig. 9G, pretreatment of the cells with bexarotene or 9-cis-RA clearly enhanced RXRα/PPARγ interaction in TNF-α-stimulated arterial endothelial cells.

Additional functional studies under physiological flow conditions were then carried out to establish RXRα/PPARγ interaction in the inhibitory effects elicited by RXRα agonists. For this purpose we first evaluated the effect of different selective PPAR ligands on TNF-α-induced mononuclear cell/HUAEC interactions (Supplemental Fig. 1). Based on these results, suboptimal concentrations of each PPAR ligand were combined with those of bexarotene or 9-cis-RA (Fig. 2). At 0.3 μM none of the RXR agonists was able to inhibit TNF-α-induced mononuclear cell/endothelium interactions (Fig. 2). When 1 μM PPARδ (GW7647) or PPARβ ligand (GW501516) was combined with 0.3 μM bexarotene or 9cis-RA, TNF-α–induced mononuclear leukocyte rolling and adhesion were not significantly affected (Fig. 10A–D). In contrast, significant reductions in these parameters were achieved when RXR agonists were combined with 1 μM rosiglitazone (Rosi), with mononuclear cell adhesion having been reduced by 61–65% (Fig. 10E, 10F). Thus, taken together these results suggest that RXRα agonists combined with PPARγ ligands can promote a synergic anti-inflammatory response.

Bexarotene inhibits leukocyte/endothelial cell interactions in vivo

To extend the functional data obtained in vitro to an in vivo model, intravital microscopy was used to examine leukocyte trafficking in the mouse cremasteric microcirculation. Stimulation with TNF-α for 4 h induced a significant enhancement of leukocyte adhesion to the cremasteric arterioles that was dose-dependently inhibited by bexarotene (Fig. 11A). Significant reductions were achieved at 30 mg/kg (60% inhibition). In the postcapillary venules of the same animals, 4 h exposure to TNF-α also induced a significant increase in venular leukocyte rolling flux, adhesion, and emigration (Fig. 11). As found in arterioles, pretreatment with bexarotene significantly reduced TNF-α–induced leukocyte/endothelial cell interactions at the highest dose tested (Fig. 11). The administration of bexarotene alone did not affect basal responses. Both arteriolar and venular diameters, as well as wall shear rates, were not affected by these treatments (Table 1). Finally, immunohistochemical analysis of the cremasteric microvasculature showed that stimulation with this cytokine caused a substantial increase in VCAM-1 and ICAM-1 expression within the cremasteric arterioles. Pretreatment of mice with bexarotene (30 mg/kg) decreased TNF-α–induced CAM expression (Fig. 11E, 11F). Similar responses were observed in postcapillary venules (data not shown).

Discussion

Selective RXR agonists are being developed for cancer therapy and are promising agents for the treatment of metabolic diseases (11). Additionally, some reports suggest that RXRα activation can also exert anti-inflammatory properties (20, 30–32), and it has proven effective in murine models of atherosclerosis (18, 19). Despite

FIGURE 6. RXR agonists diminish TNF-α–induced p38 MAPK, ERK1/2, and JNK activation in arterial endothelial cells. HUAECs were pretreated for 24 h with 1 μM bexarotene (BEX) or 9-cis-RA and then stimulated with TNF-α (10 ng/ml, 30 min), p38 MAPK (A), ERK1/2 (B), and JNK (C) activation was determined by Western blotting. Results are the means ± SEM of four independent assays and are expressed as fold increase of the phosphorylated protein versus the nonphosphorylated protein. Representative gels are also shown. *p < 0.05 relative to values in the control group. †p < 0.05 relative to values in the TNF-α–stimulated group.
these findings, the effect of rexinoids on mononuclear leukocyte recruitment is largely unknown. In the present study, to our knowledge, we have demonstrated for the first time that RXR agonists inhibit mononuclear leukocyte/endothelial cell interactions induced by TNF-α. This effect seems to be mediated via RXRα activation through the inhibition of proinflammatory CAM expression and chemokine release in primary cultures of human arterial endothelial cells. Additionally, we have also provided evidence that the inhibition of MAPKs and NF-κB activation are underlying mechanisms of this negative regulation exerted by RXR agonists. Finally, we have also revealed that the inhibitory effects of RXR agonist on TNF-α–induced mononuclear leukocyte/endothelial interactions are dependent on PPARγ activation.

TNF-α is involved in endothelial dysfunction and is recognized as a major risk factor in the initiation and progression of atherosclerotic lesion formation and the subsequent development of cardiovascular disease. TNF-α has been shown to regulate RXRα expression or its subcellular localization in liver and kidney (27, 28, 33). More recently, Giaginis et al. (34) have described that low levels of RXRα and PPARγ expression correlate with advanced carotid atherosclerosis in humans. In this study, we report that RXRα is constitutively expressed in human arterial endothelial cells, but its expression is dramatically downregulated after TNF-α exposure. Independently, under dynamic flow conditions in vitro, the pretreatment of endothelial cells with RXR agonists resulted in significant reductions in mononuclear leukocyte/arterial endothelium interactions evoked by TNF-α. Since three different RXR isotypes have been described, namely RXRα, RXRβ and RXRγ (35), in an attempt to dissect whether RXRα was the isotype involved on the observed effects, we next silenced its expression. Indeed, lack of RXRα expression blunted the inhibitory response elicited by RXR agonists, suggesting that activation of this subtype causes the impaired mononuclear cell arrest on human arterial endothelium induced by TNF-α.

This decrease in the mononuclear leukocyte/arterial endothelium adhesive interactions promoted by RXR agonism may imply the modulation of expression and/or function of CAM. The arterial endothelium has been shown to express the same CAMs as those
expressed in the venular endothelium (36–38). However, whereas in vivo leukocyte/endothelial cell interactions in postcapillary venules are provoked by a wide range of stimuli, arteriolar leukocyte adhesion is only induced by certain risk factors for atherosclerosis, including TNF-α (39). We have found that in human arterial endothelium 9-cis-RA and bexarotene are capable of inhibiting TNF-α–induced VCAM-1 and ICAM-1 increased expression. That notwithstanding, there are controversial in vitro data regarding the ability of retinoic acid to regulate CAM expression, albeit in other cell types (40–42). Whereas Gille et al. (40) showed that all-trans-retinoic acid inhibited VCAM-1, although not ICAM-1, expression induced by TNF-α in dermal microvascular endothelial cells, other studies with SK-N-SH cells, a human neuroblastoma cell line, have shown that the ICAM-1 gene is upregulated by retinoic acid in an RAR-dependent fashion (42). Different evidence can explain these discrepancies with our results. First, retinoic acid can activate other nuclear hormone receptors than RXRa. Therefore, it is likely that the increased ICAM-1 expression was not due to RXRα activation. Second, although 9-cis-RA can activate both RAR and RXR nuclear receptors, the effects displayed by this agonist are similar to those exerted by bexarotene, an RXR-specific ligand. Third, knocking down RXRα undermines the inhibition of TNF-α–induced ICAM-1 and VCAM-1 expression by these agonists. Finally, and in line with our observations, it has also been reported that RXR agonists can directly inhibit high glucose-induced oxidative stress in human endothelial cells, suggesting a role for RXR activation in vasculoprotection (20). As opposed to endothelium, RXR agonists had no effect on CD11b/CD18 expression induced by TNF-α in human monocytes.

Little is known regarding the impact of RXR activation on human chemokine release. It has been reported that retinoid acid suppressed IL-1β–induced RANTES, MCP-2, MIP-1α, and MIP-1β release in human chondrocytes (43). Additionally, all-trans-retinoic acid and 9-cis-RA were able to attenuate IL-4–induced eotaxin generation in a human bronchial epithelial cell line (44). It is well established that stimulation of vascular endothelial cells with TNF-α results in the production of a wide array of leukocyte-recruiting chemokines (4–5). Among them, MCP1/CCL2 and GROα have been shown to play a critical role in mononuclear leukocyte arrest and to participate in the progression of atherosclerotic lesions (4–6). In the present study, bexarotene and 9-cis-RA inhibited the release of GROα and MCP-1 elicited by TNF-α, an effect again blunted in cells lacking RXRα. Therefore, RXRα activation decreased cytokine-induced mononuclear arrest through inhibition of chemokine generation and release.
Accumulating data indicate that MAPK signaling pathways are modulated by retinoid acid and RXR ligands (45–49). Not surprisingly, we have found that RXR agonists downregulate the MAPKs cascade by inhibiting the phosphorylation of p38 MAPK, ERK1/2, and JNK induced by TNF-α in human arterial endothelial cells. Because previous studies have found associations between reduced levels of RXRα and phosphorylation of JNK (45, 46), it is tempting to speculate that the downregulation of RXRα induced by TNF-α could be due, among other factors, to activation of JNK-dependent pathways. The activation of different components of the MAPK family is associated with the activation of NF-κB (9), and mobilization of NF-κB into the nucleus activates the transcription of genes that encode endothelial CAM expression and chemokine synthesis (10). In our study, RXR agonists blocked both the activation of NF-κB as well as its translocation into the nucleus. RXRα-mediated pathways could also be inhibited due to direct interaction between NF-κB p65 and the RXRα DNA binding domain and thus prevent the binding of RXRα to consensus DNA sequences (50). In this context, previous findings have shown that the inhibition of IL-12 production in macrophages by RXR agonists can occur through physical associations between RXR and NF-κB (51). Consequently, hepatocyte RXRα deficiency results in NF-κB activation, as well as proinflammatory cytokine production, which in part may contribute to alcohol-induced liver damage (52).

RXRs are common heterodimerization partners of other nuclear receptors, and it is known that PPARs form permissive RXR heterodimers, which in some cases synergistically respond to both agonists, RXR and the partner receptor (53). PPARα, PPARβ, or PPARγ activation can downregulate the expression of CAMs, proinflammatory genes, and reduce leukocyte/endothelial cell interactions (24, 29). In the present study, we have first shown that whereas gene silencing of PPARα or PPARβ did not reverse the reduction in TNF-α–induced mononuclear leukocyte/arterial endothelium interactions caused by RXR agonists, PPARγ knockdown blunted this inhibitory effect. Second, when suboptimal concentrations of PPARα or PPARβ and RXR agonists were combined, no effect in TNF-α–induced mononuclear leukocyte rolling was observed (54). The mechanisms underlying these findings are not well understood, but they suggest that the combination of RXR and PPAR agonists may have additive or synergistic effects on the inhibition of mononuclear leukocyte/endothelial cell interactions.
recruitment was observed. Conversely, coincubation of HUAECs with suboptimal concentrations of RXRa, PPARa, or PPARγ agonists on TNF-α–induced mononuclear leukocyte adhesive effects. HUAECs were pretreated for 20 h with 0.3 μM bexarotene (BEX) or 9-cis-RA in combination or not with 1 μM RXRa agonist (GW7647), PPARβ agonist (GW501516), or PPARγ agonist (rosiglitazone, Rosi) and then stimulated with TNF-α (10 ng/ml, 4 h). Leukocyte rolling (A, C, E) and adhesion (B, D, F) were quantified. Data are means ± SEM of five to six independent experiments per group. *p < 0.05 relative to values in the control group, †p < 0.05 relative to values in the TNF-α–stimulated cells.

**FIGURE 10.** Effect of combined suboptimal concentrations of RXRa agonist with PPARa, PPARβ, or PPARγ agonists on TNF-α–induced mononuclear leukocyte adhesive effects. The Journal of Immunology 11

Although previous reports have shown that either the activation of PPARα or PPARβ led to decreased neutrophil/endothelial cell interactions and CAM expression, albeit in human venous endothelial cells (22, 55), RXR inhibitory effects on mononuclear leukocyte/arterial endothelium seemed to be independent of PPARα or PPARβ activation. The reasons for this apparent discrepancy might be due to the subtype of leukocyte studied (neutrophils versus mononuclear cells) or to the decreased responsiveness of the arterial endothelium to activation of these nuclear receptors. In this context, it was necessary to increase 100-fold the concentration of the PPARβ agonist to significantly diminish TNF-α–induced mononuclear cell arrest (Supplemental Fig. 1). Consequently, it is likely that arterial mononuclear cell recruitment repression by RXRs does not require PPARα or PPARβ activation.

Finally, and in agreement with the in vitro data, intravital microscopy within the murine cremasteric microcirculation revealed that bexarotene administration inhibited TNF-α–induced leukocyte-endothelial cell interactions and CAM expression. These observations appear to be in contrast to the results obtained by Núñez and colleagues (30) in which mice lacking RXRa in myeloid cells exhibit impaired recruitment of leukocytes to sites of inflammation. Administration of an agonist of RXR/PPARγ heterodimer did not increase chemokine release in wild-type macrophages (56), and in our study bexarotene did not affect CD11b/CD18 expression in TNF-α–stimulated human monocytes. Additionally, the claimed defects in leukocyte migration did not involve RXRa hetero-
dimerization with PPARγ since in the thioglycollate-induced peritonitis model neither leukocyte recruitment nor cytokine and chemokine content in the peritoneal exudate were affected in myeloid PPARγ knockout mice. Nevertheless, knockout phenotypes do not always mirror receptor antagonism, and the capability of bexarotene and other RXR agonists to decrease CAM expression and/or to impair macrophage infiltration in other murine models of vascular disease has been previously reported (19, 57). Furthermore, the same group has recently shown that murine macrophages lacking heterodimeric nuclear receptors RXRα or PPARγ expression led to severe glomerular inflammation associated with massive macrophage infiltration (30). This contention adds further support to our findings and suggests that the in vitro and in vivo effects of RXRα ligands require functional PPARγ to exert their anti-inflammatory activity.

In conclusion, to our knowledge, this is the first report to demonstrate that RXR activation leads to the inhibition of mononuclear leukocyte recruitment under dynamic conditions in vitro and in vivo. Consistent with these findings, these effects appear to be mediated through the inhibition of p38/ERK/JNK MAPKs and NF-κB activation, which causes the subsequent downregulation of endothelial CAM expression and chemokine release. Activation of PPARγ is required for RXR agonist-induced arterial anti-inflammatory responses. Therefore, this study also suggests that RXRα/PPARγ li-

### Table I. Hemodynamic parameters

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>TNF-α</th>
<th>TNF-α Plus Bexarotene</th>
<th>Bexarotene</th>
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</thead>
<tbody>
<tr>
<td>Arteriolar diameter (µm)</td>
<td>38.9 ± 1.3</td>
<td>38.41 ± 3.1</td>
<td>36.25 ± 1.9</td>
<td>37.83 ± 1.08</td>
</tr>
<tr>
<td>Arteriolar shear rate (s⁻¹)</td>
<td>700 ± 31.7</td>
<td>854.2 ± 61.7</td>
<td>805 ± 70.0</td>
<td>849.0 ± 36.22</td>
</tr>
<tr>
<td>Venular diameter (µm)</td>
<td>34.3 ± 1.7</td>
<td>37.1 ± 2.1</td>
<td>35.60 ± 0.8</td>
<td>35.2 ± 1.2</td>
</tr>
<tr>
<td>Venular shear rate (s⁻¹)</td>
<td>407.61 ± 61.9</td>
<td>483.24 ± 36.6</td>
<td>499 ± 0.9</td>
<td>471.0 ± 62.25</td>
</tr>
</tbody>
</table>

Arteriolar and venular diameter and shear rate (means ± SEM, in animals used for intravital microscopy studies) were measured 4 h after intrascrotal injection of vehicle or TNF-α (0.5 µg/mouse) in animals untreated or pretreated with bexarotene (30 mg/kg, 20 h prior TNF-α injection).
glands may constitute a new therapeutic tool in the control of the inflammatory process associated with cardiovascular disease. Furthermore, the dual administration of RXRs and PPARγ ligands could permit the administration of lower doses of either agonist reducing the appearance of further associated side effects.

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Disclosures
The authors have no financial conflicts of interest.

References

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