Activation of the Retinoid X Receptor modulates Angiotensin II-induced smooth muscle gene expression and inflammation in vascular smooth muscle cells

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ABSTRACT

The retinoid X receptor (RXR) partners with numerous nuclear receptors, such as the PPAR family, LXR, and FXR. While each heterodimer can be activated by specific ligands, a subset of these receptors, defined as permissive nuclear receptors, can also be activated by RXR agonists known as rexinoids. Many individual RXR heterodimers have beneficial effects in vascular smooth muscle cells (SMCs). Since rexinoids can potently activate multiple RXR pathways, we hypothesized that treating SMCs with rexinoids would more effectively reverse the pathophysiologic effects of angiotensin II than an individual heterodimer agonist. Cultured rat aortic SMCs were pre-treated with either an RXR agonist (bexarotene or 9-cis retinoic acid) or vehicle (DMSO) for 24 hours prior to stimulation with angiotensin II. Compared to DMSO, bexarotene blocked angiotensin II-induced SM contractile gene induction (calponin and smooth muscle-α-actin) and protein synthesis (3H-leucine incorporation). Bexarotene also decreased angiotensin II-mediated inflammation, as measured by decreased expression of monocyte chemoattractant protein–1 (MCP-1). Activation of p38 MAP kinase, but not ERK or Akt was also blunted by bexarotene. We compared bexarotene to 5 agonists of nuclear receptors (PPARα, PPARγ, PPARδ, LXR, and FXR). Bexarotene had a greater effect on calponin reduction, MCP-1 inhibition and p38 MAP kinase inhibition than any individual agonist. PPARγ knockout cells demonstrated blunted responses to bexarotene indicating that PPARγ is necessary for the effects of bexarotene. These data demonstrate that RXR is a potent modulator of angiotensin II-mediated responses in the vasculature, partially through inhibition of p38.
INTRODUCTION
As a dominant cell type within the vasculature, vascular smooth muscle cells (SMCs) are important to maintain vascular tone and are key initiators of vascular disease. In a healthy adult artery, SMCs are quiescent and express a family of SM-specific proteins, including calponin, SM-α-actin, and SM myosin heavy chain. In response to multiple environmental cues, such as mechanical injury or growth factors, SMCs can undergo tremendous phenotypic changes. (Owens et al., 2004) For example, in the setting of angioplasty or after stimulation with PDGF-BB, SMCs lose expression of differentiation markers, proliferate, and migrate from the media leading to neointima formation. In contrast, in the setting of angiotensin II-induced hypertension, SMCs undergo hypertrophy and increase expression of SMC-specific markers; eventually, this process will lead to thickening of the arterial media and increased vascular resistance. (Mack, 2011) Angiotensin II (AII) is an octapeptide that is closely linked to a variety of cardiovascular diseases. After binding to the angiotensin type 1 receptor (AT₁R) on SMCs, AII causes an acute increase in intracellular calcium leading to SMC contraction. Over hours to days, via activation of multiple downstream pathways including extracellular signal related kinase (ERK) and p38 MAP kinase, AII signaling results in increased expression of smooth muscle specific genes, hypertrophy, and expression of inflammatory cytokines. (Mehta and Griendling, 2007) Chronically, AII-induced vascular remodeling increases vascular resistance and promotes chronic hypertension. In many clinical trials, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) have significant cardiovascular benefits that may be partially due to the effects on vascular remodeling. (Mancia et al., 2013)
Retinoid X receptors (RXRs), members of the nuclear receptor superfamily, are common heterodimer partners with other nuclear receptors, such as Retinoic Acid Receptor (RAR), peroxisome proliferator activated receptors (PPARs), and liver X receptor (LXR). These heterodimers act as ligand-activated transcription factors. Two examples of specific nuclear receptor agonists are thiazolidinediones, selective PPARγ-RXR agonists, and fibrates, selective PPARα-RXR agonists. In contrast, specific RXR agonists (rexinoids) have the potential to activate a large subset of heterodimers, including all PPAR isoforms, LXR, and farnesoid X receptor (FXR). Heterodimers activated by both rexinoids and nuclear receptor-specific ligands are termed permissive heterodimers. Other RXR heterodimers, such as RAR-RXR and vitamin D receptor (VDR)-RXR, cannot be activated by rexinoids alone and are termed non-permissive heterodimers. In addition to activating RXR heterodimers, in cancer cells and monocytes, rexinoids have been shown to activate pathways regulating growth and inflammation via RXR-RXR homodimers. Rexinoids have been shown to improve insulin sensitivity in animal models of diabetes, likely through activation of PPARγ. Multiple preclinical studies have shown rexinoids have anti-tumor efficacy and bexarotene, a selective RXR agonist, is FDA-approved for cutaneous T cell lymphoma. Recently, bexarotene was shown to be protective in a mouse model of Alzheimer’s disease by increasing clearance of amyloid plaques.

There is accumulating data that permissive nuclear receptor agonists may activate potent vasculoprotective pathways. In SMCs, individual nuclear receptors have been
shown to exert important biological effects. For instance, activation of PPARγ by rosiglitazone has been shown to decrease angiotensin II-induced SMC hypertrophy. (Benkirane et al., 2006) In contrast, PPARδ agonists decrease SMC senescence associated with angiotensin II (Kim et al., 2011) while LXR agonists decrease expression of the AT1R receptor. (Imayama et al., 2008) The effects of rexinoids on SMCs have not been studied; however, in endothelial cells, bexarotene inhibits TNFα-induced adhesion molecule expression and decreases mononuclear cell recruitment. (Sanz et al., 2012)

Activating multiple nuclear receptors may represent a potent approach for modulating SMC phenotype. By activating multiple nuclear receptors, rexinoids may be able to engage distinct pathways (ie. PPARγ- and LXR-induced pathways) which cooperate to promote enhanced responses compared to activation of a single nuclear receptor. In macrophages, PPARγ and LXR control overlapping and non-overlapping anti-inflammatory pathways. (Ogawa et al., 2005) In hepatocytes, gene profiling revealed that rexinoids, PPARα agonists, and LXR agonists activate both common pathways and unique targets. (Boergesen et al., 2012) We therefore hypothesized that rexinoids would more potently inhibit the biological effects of angiotensin II on SMCs compared to an individual nuclear receptor agonist.

**MATERIALS AND METHODS**

**Materials** Eagle’s Minimal Essential Media (EMEM, Mediatech, Mannassas VA) and Fetal Calf Serum (Hyclone, South Logan UT) were used for culturing VSMCs. For
transient transfections, cells were placed in Opti-MEM (Life Technologies, Carlsbad, CA). Bexarotene (Biovision, Milpitas CA), 9-cis retinoic acid (Sigma, St Louis MO), pioglitazone (Cayman Chemical, Ann Arbor MI), fenofibrate (Cayman), T0901317 (Cayman), GW 501516 (Enzo, Farmingdale NY), 6-ECDCA (Cayman), PD 98059 (EMD Millipore, Billerica MA) and SB 203580 (EMD Millipore, Billerica MA) were all dissolved in dimethyl sulfoxide (DMSO, Sigma, St Louis MO).

**SMC Isolation and Culture** Rat SMCs were isolated and cultured as described previously. (Furgeson et al., 2010) Briefly, thoracic aortas were dissected from male Harlan Sprague-Dawley rats (250–300 g) and incubated in EMEM containing 2 mg/ml collagenase for 1 h at 37°C. The adventitia was removed, and the aortas were minced and incubated in the MEM collagenase solution at 37°C for 2 h. The isolated cells were plated at a density of $1 \times 10^4$ cells/ml culture media (EMEM) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum in 35-mm culture dishes. Cells were passed by trypsinization and used between passage numbers 7 to 15. For all experiments, cells were growth arrested in EMEM with 0.1% calf serum the day after plating.

**Quantitative RT-PCR** Total RNA was isolated from SMCs using the QIAshredder and RNeasy plus kits (Qiagen, Valencia CA) and first strand cDNA was made using the iScript cDNA synthesis kit (BioRad, Hercules CA). qRT-PCR reaction was performed with SYBR Green PCR master mix (Applied Biosystems, Carlsbad CA). Sequence-specific primers are as follows: Calponin F (5’-CACCAATCATAACAAGTTCA-3’) R (5’-CTTCTCAGGCTCAAATCTCC-3’), GAPDH F (5’-
Western analysis and ELISA At the indicated times SMCs were lysed with ice-cold RIPA buffer, pH 7.4, (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 1% Na deoxycholate, 0.1% SDS, 50mM NaF, 2mM EDTA 200μM Na₃VO₄, and protease inhibitors). Solubilized proteins were centrifuged at 14,000 x g in a microcentrifuge (4°C) for 10 min. Supernatants were separated using 10% SDS- polyacrylamide gel electrophoresis and transferred to Immobilon P membranes (Millipore, Billerica MA). Membranes were blocked for 1 hour at room temperature in Tris-buffered saline (10mM Tris- HCl, pH 7.4, 140mM NaCl) containing 0.1% Tween-20 (TTBS) and 2.5% BSA (Sigma, St. Louis MO), and then incubated with 5% BSA in TTBS solution containing primary antibodies for 16 hours at 4°C. Membranes were washed in TTBS, and bound antibodies were visualized either with Horseradish Peroxidase-coupled secondary antibodies and ECL reagent (Fisher, Pittsburgh PA) or Alkaline phosphatase-coupled secondary antibodies and Lumino-
Phos WB (Thermo Scientific, Rockford IL) according to the manufacturer’s directions. Antibodies used were Calponin (Abcam, Cambridge MA), Phospho-p38, Total p38, Phospho-ERK, Total ERK, Phospho-Akt, Total Akt, Phospho-4EBP1 (Cell Signaling, Danvers MA), and Sm-α-actin and β-Actin (Sigma, St. Louis MO). Anti-rabbit and anti-mouse secondary antibodies were used (Santa Cruz, Dallas TX). ELISA was performed as previously described. (Nemenoff et al., 2008) To detect secreted MCP-1 conditioned media from SMCs 48 hours after stimulation with angiotensin II was assayed using a mouse/rat MCP-1 specific solid-phase ELISA kit according to the protocols provided (R&D Systems, Minneapolis MN). Values were normalized to total cell protein from individual cell cultures.

**Plasmids and Transient Promoter Assays** For transient transfections measuring promoter activity, a plasmid encoding 549nt upstream of the calponin initiation site ligated into a luciferase vector (PA3-Luc) was used (a gift from Joseph Miano, University of Rochester). SMCs were transiently transfected with Lipofectamine PLUS (Life) in 60-mm dishes using 2 μg of the calponin promoter-luciferase construct together with 2 μg of a plasmid encoding cytomegalovirus-β-galactosidase vector (Clontech, Mountain View CA) for normalization of transfection efficiency. Some experiments also included 2 μg of CA-MKK6 or empty vector/pCDNA (Life). Cells were incubated for 16 h in Eagle's minimal essential medium with 0.2% fetal calf serum and then placed in Eagle's minimal essential medium with 0.1% fetal calf serum with or without angiotensin II and bexarotene for 48 h. For PPAR-RE and LXR-RE reporter assays, 2 μg of reporter plasmid and 2 μg of β-galactosidase were added to SMCs in the presence of
Lipofectamine PLUS. After 5 hours, EMEM with 10% calf serum was added and media was changed at 24 hours. Agonists were added for an additional 24 hours. Transiently transfected SMCs were then washed twice with ice-cold phosphate-buffered saline and harvested in luciferase reporter lysis buffer (Promega, Madison, WI). Cell lysates were centrifuged, and the supernatants were assayed for luciferase and β-galactosidase activities as previously described. (Horita et al., 2011)

3H-Leucine Incorporation SMCs were serum restricted in 0.1%-FCS-EMEM for 24 hours then treated with bexarotene or DMSO for an additional 24 hours. Fresh drugs plus or minus angiotensin II (10⁻⁶ M) were then added to serum-free media. 3H-Leucine (1mCi/ml) was added to the media at the same time and cells were incubated for 24 hours. Cells were rinsed extensively on ice with ice-cold PBS to remove free 3H-Leucine, and then precipitated with 10% (w/v) trichloroacetic acid (TCA, Sigma) for 30min on ice. Cells were then rinsed with 10% TCA, followed by three washes with 95% ice-cold ethanol. Ethanol was removed and samples were air-dried on ice. Precipitated material was solubilized with 300 μL of SDS/NaOH solution. 3H-leucine incorporation was quantified via scintillation (Beckman).

shRNA Infection of SMCs Lentiviral packaging vectors, control plasmid (pGIPZ) and PPARγ-specific shRNA plasmid (pGIPZ-PPARγ) were purchased from Open Biosystems (GE Dharmacon, Lafayette, CO). HEK293T cells were infected with lentiviral plasmids in presence of EMEM media with 10% calf serum. The following day, media was removed, filtered through a 40 μm filter, and added to target SMCs. On the next day,
SMC media containing 2 μg/mL puromycin was added for selection. Cells were used for experiments after two passages under puromycin selection.

**Transfection with siRNA oligonucleotides** Non-targetting siRNA oligonucleotides and oligonucleotides for DUSP isoforms were purchased from Dharmacon (GE Dharmacon, Lafayette, CO). Cells were transfected with 25 nM of oligonucleotides using Dharmafect 2 (Dharmacon) for 24 hours following the manufacturer’s protocol.

**Statistical Analysis** All experiments were repeated a minimum of three times. Paired t tests were performed to compare drug treatment effect to DMSO. For multiple condition comparisons, ANOVA with Tukey’s post-test was used. All data are presented as mean +/- SE.

**Results**

**Rexinoids inhibit angiotensin II-induced SMC differentiation and SMC hypertrophy** We first determined whether bexarotene(Altucci et al., 2007), a selective rexinoid, modulated the effects of angiotensin II on expression of the SMC-specific markers, SM-α-actin (SMA) and calponin in cultured aortic SMCs. We treated SMCs with 1 μM bexarotene, a concentration achievable in humans using standard dosages.(Rizvi et al., 1999) Since bexarotene most likely causes transcription of target genes in SMCs, we treated SMCs with bexarotene for 24 hours prior to adding other agonists. To induce expression of SMC genes over a 48 hour period, we treated cells with angiotensin II 10^{-6} M. Since angiotensin II is degraded by proteases over a 48 hour period, we used the
higher concentration of angiotensin II. Bexarotene alone had no effect on expression of SMC markers. However, pre-treatment with bexarotene blocked the increase in SMC marker expression induced by angiotensin II (Figure 1A-B). As opposed to induction of SM marker genes, PDGF-BB is a known repressor of the SMC differentiation program. In contrast to effects mediated by AII, bexarotene had no effect of PDGF-BB-mediated downregulation of SM markers (data not shown). To demonstrate that these biological effects were not due to off-target effects of bexarotene, we repeated the experiments with a second chemically distinct rexinoid: 9-cis retinoic acid (alitretinoin)(Altucci et al., 2007). 9-cis retinoic acid at a concentration of 1 μM is also similar to concentrations seen in humans.(Lawrence et al., 2001) Both rexinoids inhibited the upregulation of calponin protein and mRNA after stimulation with angiotensin II (Figure 1C and 1D). A calponin luciferase reporter assay was conducted to confirm that effects of rexinoids occur at the transcriptional level. As shown in Figure 1E, bexarotene pre-treatment blocked AII-induced calponin promoter activity.

We next examined the effect of bexarotene on AII-induced SMC hypertrophy. Bexarotene inhibited angiotensin II-induced hypertrophy, as measured by protein synthesis using 3H-leucine incorporation (Figure 2A). Angiotensin II-induced hypertrophy is associated with phosphorylation and inactivation of the translation repressor protein, eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1).(Benkirane et al., 2006) Consistent with an effect on SMC hypertrophy, bexarotene pre-treatment resulted in significantly less phosphorylated, and therefore active 4E-BP1 at 20 minutes (Figure 2B). This data suggests that modulation of 4E-BP1 is one possible mechanism mediating the anti-growth effects of bexarotene.
Rexinoids negatively regulate angiotensin II-induced inflammation

As angiotensin II is also a potent SMC pro-inflammatory agent, we tested whether bexarotene affected transcription of inflammatory cytokines. As shown in Figure 3A, angiotensin II significantly increased monocyte chemoattractant protein (MCP-1/CCL-2) mRNA at three hours. While bexarotene and 9-cis retinoic acid alone had no effect on MCP-1 expression, pre-treatment with either agent blunted the increase in MCP-1 induced by AII. Furthermore, to confirm a change in MCP-1 protein, levels of MCP-1 in conditioned media were measured. Angiotensin II increased MCP-1 protein levels at 48 hours while bexarotene pre-treatment attenuated the increase in MCP-1 (Figure 3B).

Activation of p38 MAP kinase is Blunted by Rexinoids

Since p38 and ERK MAP kinases have been shown to drive smooth muscle gene expression and MCP-1 production, we next studied whether rexinoids modulated short term signaling events. As with the previous experiments, SMCs received 24 hours of bexarotene treatment prior to stimulation with angiotensin II to allow transcription and translation of RXR targets. Cells were stimulated with angiotensin II 10^{-6}M in order to correlate the short-term experiments with the 48-hour experiments. As shown in Figure 4A, angiotensin II led to a rapid induction of phospho-p38 MAP kinase, which returned to basal levels by 120 minutes after stimulation. While bexarotene pre-treatment had no effect on maximal activation of p38 at 5 minutes, there was a significant reduction after 20 minutes of angiotensin II stimulation. This same pattern was seen using a lower concentration of angiotensin (10^{-7}M, not shown). In contrast, while angiotensin II
stimulation also activated ERK and Akt, bexarotene did not inhibit either (Figure 4B&C) suggesting bexarotene selectively blocks angiotensin II-mediated p38 signaling.

To determine if the rexinoids’ effect on calponin induction was mediated via p38 inhibition we compared bexarotene pre-treatment to pre-treatment with either a MEK inhibitor (PD98059; upstream ERK inhibitor) or a p38 inhibitor (SB203580). As shown in Figure 5A and 5B, bexarotene and SB203580 both inhibited calponin induction at the mRNA and protein levels while PD98059 had no effect. Induction of p38 also, in part, mediates MCP-1 induction after angiotensin II. (Ebrahimian et al., 2011; Takahashi et al., 2008) Bexarotene and SB203580, but not PD98059, inhibited induction of MCP-1 mRNA (Figure 5C). Collectively, these data indicate that effects of RXR agonists on angiotensin II-induced SMC gene and MCP-1 expression are possibly mediated via negative regulation of p38.

MKK6 is an upstream kinase for p38 and we have previously shown that transfecting SMCs with a constitutively active MKK6 construct increases SMC gene expression. (Garat et al., 2000) Since MKK6 is a downstream effector of the angiotensin II type 1 receptor (AT₁R), it is unlikely that if bexarotene solely blocks AT₁R activity, it would retain an inhibitory effect in the presence of constitutively activated MKK6. Given that bexarotene did not inhibit p38 activation at 5 minutes, we predicted it was not blocking upstream activators of p38. To confirm this possibility, SMCs were co-transfected with a calponin promoter-luciferase reporter construct and a constitutively active MKK6 plasmid. Consistent with an inhibitory role for bexarotene downstream of AT₁R-MKK6-p38 signaling, bexarotene continued to block the effect of angiotensin II on
SMC gene expression even in the presence of active MKK6 (Figure 6A). These data raised the possibility that rexinoids upregulated phosphatases that inactivate p38.

Inactivation of p38 is controlled by several members of the dual specificity phosphatase (DUSP) family. Multiple DUSPs have been described, which have different specificities for individual MAP kinase family members. Using qRT-PCR, we tested whether bexarotene increased levels of the DUSPs reported to dephosphorylate p38, DUSPs 1, 2, 4, 5, 6, 7, 8, 9, 10, 14, 16, and 26. We found that DUSP4, DUSP5, and DUSP10 were significantly increased with bexarotene (Figure 6B). Knockdown experiments with siRNA constructs targeting each of these DUSPs did not reverse the effects of bexarotene (not shown). However, knockdown of DUSP4 was also associated with compensatory increases in other DUSPs (Supplemental Fig. 1).

Comparison of RXR Agonists to Individual RXR Heterodimer Agonists

Since rexinoids activate multiple nuclear receptors, we next compared bexarotene with individual permissive nuclear receptor agonists to determine if one or more receptor predominantly mediated the effects of bexarotene on SMCs. Bexarotene was compared to WY14613 (PPARα agonist)(Forman et al., 1997), pioglitazone (PPARγ agonist)(Sakamoto et al., 2000), GW501516 (PPARδ agonist)(Oliver et al., 2001), T0901317 (LXR agonist)(Repa et al., 2000), and 6-α-ethyl-chenodeoxycholic acid (6-ECDCA, FXR agonist)(Pellicciari et al., 2002). We first confirmed that certain agonists activated target gene transcription using reporter constructs. Pioglitazone 10 μM activated a PPARγ response element to a similar extent as bexarotene (Supplemental Figure 2A). T0901317 and bexarotene both activated LXR transcription at concentrations of 1 μM.
(Supplemental Figure 2B). Bexarotene was much more effective at inhibiting p38 activation at 20 minutes than any individual agonist (Figure 7A). Similarly, bexarotene inhibited MCP-1 and calponin induction significantly more than any individual agonist (7B-C), suggesting that bexarotene activates multiple nuclear receptor pathways.

Since PPARγ has been shown to mediate widespread effects on SMC phenotype, we tested whether PPARγ was a necessary nuclear receptor for rexinoids. We generated stable PPARγ-depleted SMCs using lentiviral infection. PPARγ-depleted SMCs displayed augmented induction of calponin after treatment with angiotensin II compared to control cells. In cells expressing control shRNA, bexarotene completely inhibited angiotensin II-mediated calponin induction; cells with PPARγ shRNA demonstrated a blunted response to bexarotene (Figure 8).

DISCUSSION

Here we demonstrate that specific RXR agonists can have selective effects on SMC phenotype. Our data indicate that bexarotene blocks many of the responses of cultured SMC to angiotensin II, including hypertrophy, induction of SM-specific genes, and production of pro-inflammatory cytokines. These effects are mediated at least in part through blocking angiotensin II-mediated activation of p38 MAP kinase. Rexinoids were able to inhibit induction of SM-genes in response to expression of a constitutively active form of the upstream p38 kinase, MKK6, indicating that the inhibitory effects do not directly target the angiotensin II receptor, but instead act downstream at the level of MAP
kinase signaling. Since rexinoids target nuclear receptors, which are ligand-activated transcription factors, this suggests the possibility that the inhibitory effects on p38 activation are mediated through increased expression of counter-regulatory phosphatases. The DUSP family of phosphatases has been shown to dephosphorylate and inactivate MAP kinase family members. We found that three separate DUSPs were increased in response to bexarotene. While silencing individual DUSPs using siRNA approaches did not reverse the inhibitory effects of bexarotene, multiple DUSPs target p38, and there is significant redundancy in these proteins. As our data suggests, silencing an individual DUSP may lead to compensation by other isoforms. This effect has been reported in cardiomyocytes, where redundancy of DUSP1 and DUSP4 was demonstrated (Auger-Messier et al., 2013). Interestingly, bexarotene did not affect the responses of SMC to PDGF. This suggests that angiotensin II and PDGF regulate different signaling pathways to mediate effects on SM gene expression.

Importantly, the ability of bexarotene to block the effects of angiotensin II on SMC was greater than activators of individual nuclear receptors. One explanation could be that rexinoids are more potent activators of target gene pathways. It is possible that higher doses of pioglitazone, for instance, may have similar effects as low dose bexarotene. However, our promoter assays did not demonstrate a greater activation with bexarotene. Given the data in other cell types, it is likely that rexinoids activate multiple nuclear receptors that cooperate to mediate the inhibitory effects observed. Consistent with this model, PPARγ agonists have been shown to repress angiotensin II-induced protein synthesis (Benkirane et al., 2006) but do not appear to regulate SM-gene expression. Thus the ability of bexarotene to inhibit both responses, suggests that
additional nuclear receptors may mediate the effects on SM gene expression. Since both overall protein synthesis and smooth muscle gene induction are important in vascular hypertrophy, rexinoids may be potent anti-hypertrophic agents. Combinatorial studies using agonists for specific receptors will be required, although the number of permissive receptors makes these studies difficult, and specific agonists are not available for all the permissive receptors. In addition, bexarotene may mediate some biological effects via activation of RXR homodimers in the vasculature. Finally, since RXR binds to other orphan nuclear receptors (such as Nur77 and pregnane X receptor/PXR), it is possible that rexinoids activate these receptors as well. While no data exist demonstrating that bexarotene activates Nur77-RXR heterodimers, other rexinoids have been shown to activate Nur77. (Ishizawa et al., 2012) The relationship between bexarotene and PXR is complex; a recent study indicated that bexarotene acts as an antagonist to PXR transcription. (Pettersson et al., 2008)

The above data raises numerous therapeutic possibilities for RXR agonists in vascular disease. Since rexinoids’ effects on angiotensin-mediated responses lies downstream of the angiotensin receptor, specifically at the level of MAP kinase signaling, it is possible that rexinoids could negatively regulate the biological effects of other cytokines and growth factors in the vasculature by acting on a common downstream pathway. Angiotensin II is clearly a key mediator of many vascular diseases, such as hypertensive vascular remodeling, post-angioplasty restenosis, transplant vasculopathy, and aortic aneurysm. (Heeneman et al., 2007; Richter et al., 2003; Valente et al., 2012) However, in all of those conditions, other vasoconstrictors, growth factors, cytokines, and mechanical forces also contribute to the disease process. Therefore, our study has
potential important implications in that rexinoids may possess increased efficacy to inhibit a broader spectrum of agents compared to specific angiotensin receptor blockers.

The likelihood that rexinoids will modulate the phenotype of several cell types may also predict a potent therapeutic effect in many vascular diseases. Our study focused on the effects of RXR agonists in SMCs, but there is data that rexinoids may exert beneficial effects in other cardiovascular cell types. In endothelial cells, rexinoids reduce expression of adhesion molecules and decrease inflammatory cell recruitment in atherosclerosis. PPARγ appears to be the predominant target of rexinoids in endothelial cells. (Sanz et al., 2012) Due to LXR activation in macrophages, rexinoids also alleviate atherosclerosis. (Lalloyer et al., 2006) Other cell types in the cardiovascular system, such as fibroblasts and cardiomyocytes have not been comprehensively examined. Given the variety of nuclear receptors and different cell types, rexinoids may be more effective than individual agonists in a variety of vascular diseases. Vascular disease in humans often involves multiple pathologic events; for example, restenosis usually occurs in the background of an atherosclerotic vessel. Therefore, successfully treating vascular remodeling in humans may require therapies that inhibit multiple extracellular signals in many cell types.

The large number of targets raises the possibility that rexinoid therapy may increase the likelihood of adverse effects. For example, bexarotene is associated with hypertriglyceridemia, possibly via activation of LXR-RXR heterodimers in the liver. (Lalloyer et al., 2009) Consequently, further studies may also test whether a combination of two agonists (such as PPARα and PPARγ) may effectively mediate positive effects without negative effects (LXR induced hypertriglyceridemia). Identifying
cellular and molecular targets of rexinoids may enable activation of vasculoprotective pathways while avoiding targets that raise triglycerides.

In conclusion, we have shown that RXR agonists significantly inhibit angiotensin II-induced phenotypic change in cultured SMCs. A summary of our hypothetical model is shown in Figure 9. Our data suggests that rexinoids activate multiple nuclear receptors in SMCs, including PPARγ. By activating multiple nuclear receptors, rexinoids inhibit p38 activation, SMC hypertrophy, and SMC inflammation. Due to large number of cellular and molecular RXR targets, identifying specific nuclear receptors and downstream pathways may reveal novel, potent pathways for treating a variety of vascular diseases.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Lehman, Horita, Ostriker, Weiser-Evans, Nemenoff, Furgeson

Conducted experiments: Lehman, Montford, Furgeson

Performed data analysis: Lehman, Furgeson

Wrote or contributed to the writing of the manuscript: Lehman, Weiser-Evans, Nemenoff, Furgeson
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References


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**FOOTNOTES**

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FIGURE LEGENDS

Figure 1. RXR activation inhibits angiotensin II-induced smooth muscle gene expression. SMCs were pre-treated with either bexarotene (1 μM) or 9-cis retinoic acid (1 μM) for 24 hours and then stimulated with DMSO or AII (10^{-6} M) for 3 hours (RNA) or 48 hours (protein and luciferase assay). (A&B). Western blot demonstrating that bexarotene inhibits angiotensin II-mediated calponin (A) and SM-α-actin (SMA) (B) induction. (C&D). Western blot (C) and qRT-PCR (D) showing that 9-cis retinoic acid and bexarotene inhibit induction of calponin protein (C) and mRNA (D). For all Westerns, β-Actin was used as a loading control. Representative blots are shown; fold changes in densitometry measurements ± SEM are shown in graphs. (E). Calponin-luciferase reporter assay demonstrating bexarotene blocks angiotensin II-induced transcription of calponin. DMSO=dimethyl sulfoxide, BEX=bexarotene, 9-cis RA = 9-cis retinoic acid, SFM=serum-free media, AII=angiotensin II. *p<0.05 vs SFM DMSO.

Figure 2. Bexarotene inhibits smooth muscle hypertrophy. (A). SMCs were pre-treated with bexarotene for 24 hours and 3H leucine was added for an additional 24 hours. 3H leucine incorporation was measured as described in Materials and Methods. (B). After bexarotene pre-treatment, cells were collected at indicated time points after angiotensin II treatment and subjected to Western blotting for P-EBP-1. β-Actin was used as a loading control. Representative blot shown; fold changes in densitometry measurements ± SEM are shown in graph. * p<0.05 vs DMSO.

Figure 3. Rexinoids inhibit cytokine expression after stimulation with angiotensin II. Cells were pre-treated for 24 hours with bexarotene or 9-cis retinoic acid and stimulated
with angiotensin II for 3 hours (mRNA) or 48 hours (protein). (A). Total RNA was isolated and qPCR conducted for MCP-1 mRNA. Shown are mRNA absolute copy number normalized to GAPDH. Means ± SEM; n=3 independent experiments. (B). Conditioned media was collected and assayed for MCP-1 protein levels normalized to total protein. Shown are means ± SE; * p<0.05 vs DMSO.

**Figure 4. Bexarotene inhibits p38 activation after stimulation with angiotensin II.**
Cells were treated for 24 hours with bexarotene prior to stimulation with angiotensin II (10^{-6} M) for indicated times. (A-C). Left panels: Representative western blots for phospho- and total p38 (A), phospho- and total ERK (B), and phospho- and total Akt (C). β-actin was used as a loading control. N=3 independent experiments. Right: Fold changes in densitometry measurements ± SEM are shown in the graphs. * p<0.05 vs AII DMSO at 20 minutes.

**Figure 5. Bexarotene modulates SMC phenotype via changes in p38.** SMCs were pre-treated with bexarotene, a p38 inhibitor (SB203580, 10 μM), or a MEK inhibitor (PD98059, 10 μM) followed by stimulation with angiotensin II. Total RNA and whole cell lysates were isolated 3 hrs and 48 hrs after angiotensin II stimulation, respectively. (A). qPCR analysis for calponin mRNA. Shown are mRNA absolute copy numbers normalized to GAPDH. (B). Western analysis for calponin protein levels; shown is a representative Western from three independent experiments; β-actin was used as a loading control. (C) qPCR for MCP-1 mRNA. Shown are mRNA absolute copy numbers normalized to GAPDH. * p<0.05 vs. AII DMSO.
Figure 6. Bexarotene inhibits p38 activation downstream of MKK3/6. (A). SMCs were co-transfected with constitutively active MKK3/6 or control (pCDNA) and calponin-luciferase reporter. After pre-treatment with bexarotene, cells were stimulated for 48 hours with angiotensin II. Luciferase activity normalized to β-galactosidase was determined. p=0.06 ca-MKK3/6-DMSO vs caMKK3/6-BEX. B. qRT-PCR showing increased levels of DUSP4, DUSP5, and DUSP10. * p<0.05 vs. DMSO.

Figure 7. Bexarotene inhibits angiotensin II-mediated calponin and MCP-1 induction by recruitment of multiple RXR heterodimers. SMCs were pre-treated with bexarotene, pioglitazone (PPARγ agonist, 10 μM), WY-14643 (PPARα agonist, 1 μM), GW-501516 (PPARδ agonist, 1 μM), TT0901317 (LXR agonist, 1 μM) and 6-ECDCA (FXR agonist, 1 μM). (A). WCL were collected 20 minutes after angiotensin II stimulation. Left: Western analysis for phospho-p38 protein levels; shown is a representative Western from three independent experiments; β-actin was used as a loading control. Right: Fold changes in densitometry measurements ± SEM are shown in the graphs. (B&C). Total RNA was collected 3 hours after stimulation with angiotensin II. qPCR analysis for calponin (B) and MCP-1 (C) mRNAs. Shown are mRNA absolute copy numbers normalized to GAPDH. * p<0.05 vs. AII DMSO.

Figure 8. Bexarotene inhibits angiotensin II-mediated calponin induction partly through activation of PPARγ.
Cells expressing control shRNA or PPARγ shRNA were pre-treated for 24 hours with bexarotene prior to 48 hour treatment with angiotensin II. Shown is a representative Western blot for calponin. Densitometry measurements of both cell types treated with bexarotene and angiotensin II is shown on the right. ** p<0.01.

Figure 9. Hypothetical model demonstrating proposed relationship between bexarotene treatment and effects on SMC phenotype.

Bexarotene activates multiple nuclear receptors, including PPARγ. The combinatorial activation of multiple receptors increases expression of multiple DUSPs, which are negative regulators of p38, thereby inhibiting the pro-hypertrophic and pro-inflammatory effects of angiotensin II.
FIGURE 1

A. Calponin

B. SM-α-Actin

C. Calponin

D. Calponin/GAPDH

E. Calponin/LUC/β-gal
FIGURE 2
FIGURE 3
FIGURE 4
**FIGURE 5**

A. Graph showing Calponin mRNA normalized to GAPDH expression levels with various treatments.

B. Western blot images showing Calponin and β-Actin expression levels under different conditions.

C. Bar graph showing the fold change in MCP-1 mRNA post-Ang with different treatments.
FIGURE 6

A. 

B. 

DUSP 4 mRNA/β-Actin

DUSP 5 mRNA/β-Actin

DUSP 10 mRNA/β-Actin

FIGURE 6
FIGURE 7
A. Calponin and β-Actin Western Blot Images

B. Bar Graph showing Calponin/β-Actin ratio

FIGURE 8
FIGURE 9

Bexarotene → RXR heterodimers (including PPARγ-RXR) → Increase in DUSPs → Decreased SM-markers and SMC hypertrophy, Decreased MCP-1
Supplemental Figure 1. DUSP4 depletion leads to compensatory increases in other DUSP family members.
Cells were transfected with non-targeting siRNA or DUSP4-specific siRNA and cells were collected for RNA 48 hours later. DUSP4 knockout cells demonstrated increased DUSP1 (A) and DUSP6 (B) levels.

**Supplemental Figure 2. Effects of nuclear receptor agonists on promoter assays.**

Cells were transfected with either PPAR-RE luciferase plasmids (A) or LXR-RE luciferase plasmids (B) for 24 hours. Cells were stimulated for an additional 24 hours and collected. Bexarotene 1 μM and pioglitazone 10 μM lead to similar activation of PPAR-RE transcription (A). Bexarotene 1 μM and T0901317 both significantly activate LXR-RE (B). BEX = bexarotene, PIO = pioglitazone.