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RESEARCH PAPER

Combined treatment with bexarotene and rosuvastatin reduces angiotensin-II-induced abdominal aortic aneurysm in apoE$^{-/-}$ mice and angiogenesis

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BACKGROUND AND PURPOSE

Abdominal aortic aneurysm (AAA) is a degenerative vascular disease associated with angiogenesis. Bexarotene is a retinoid X receptor (RXR) ligand with anti-angiogenic activity. Statins also exert anti-angiogenic activity and activate PPARs. Because RXR ligands form permissive heterodimers with PPARs and a single anti-angiogenic drug may not be sufficient to combat the wide array of angiogenic factors produced during AAA, we evaluated the effect of combined low doses of bexarotene and rosuvastatin in a mouse model of AAA.

EXPERIMENTAL APPROACH

The effect of the combined treatment was investigated in a murine model of angiotensin II-induced AAA in apoE$^{-/-}$ mice. This combination therapy was also evaluated in *in vivo* (Matrigel plug assay) and *in vitro (*endothelial cell differentiation assay) models of angiogenesis as well as the underlying mechanisms involved.

KEY RESULTS

Co-treatment with bexarotene plus rosuvastatin reduced aneurysm formation, inflammation and neovascularization compared with each single treatment. In HUVEC, the combination of suboptimal concentrations of bexarotene and rosuvastatin inhibited angiotensin II-induced morphogenesis, proliferation and migration. These effects were accompanied by diminished production of pro-angiogenic chemokines (CXCL1, CCL2 or CCL5) and VEGF, and seemed to be mediated by RXRα/PPARα and RXRα/PPARγ activation. This combined therapy reduced the activation of members of the downstream PI3K pathway (Akt/mTOR and p70S6K1) *in vivo* and *in vitro*.

CONCLUSIONS AND IMPLICATIONS

The combination of RXR agonists with statins at low doses synergistically interferes with the signalling pathways that modulate inflammation and angiogenesis and may constitute a new and safer therapeutic treatment for the control of AAA.

Abbreviations

Akt, V-Akt murine thymomaviral oncogene homologue kinase; Ang-II, angiotensin-II; CCL2, (C-C motif) ligand 2 which in mice represents the human monocyte chemotactic protein-1 (MCP-1); CCL5, (C-C motif) ligand 5 which in mice represents the human chemokine regulated on activation normal T-cell expressed and secreted (RANTES); CXCL1, (C-X-C motif) ligand 1 which in mice represents the human chemotactic growth-related oncogene-α (GRO-α); mTOR, mammalian target of the rapamycin; p70S6k1, 70 kDa ribosomal protein S6 kinase 1; RXR, retinoid X receptor

Tables of Links

TARGETS	
GPCRs[a]	**Catalytic receptors**[c]
AT_1 receptor	VEGFR1
CCR1	VEGFR2
CCR5	**Enzymes**[d]
Nuclear hormone receptors[b]	Akt (PKB)
PPARα	CYP3A4
PPARβ	CYP2C9
PPARδ	CYP2C19
PPARγ	mTOR
RXRα	p70S6k1
RXRβ	

LIGANDS	
Angiotensin II	IL-8 (CXCL8)
Bexarotene	Rosuvastatin
CCL2 (MCP-1)	SB 225002
CCL5 (RANTES)	Simvastatin
CXCL1	TNF-α
EXP3174	UCB35625
IL-6	VEGF

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d]Alexander *et al.*, 2013a,b,c,d).

Introduction

Abdominal aortic aneurysm (AAA) is a degenerative disease of the aorta that mainly affects people over the age of 65 (Golledge *et al.*, 2006). AAA can be detected in humans by non-invasive imaging techniques; however, nowadays, there are no pharmacological treatments to prevent the progression of this disease (Golledge *et al.*, 2006).

In recent years, new microvessel formation or angiogenesis in aortic aneurysmal disease has been associated with the risk of rupture and complications (Choke *et al.*, 2006). Angiotensin-II (Ang-II) is the main effector peptide of the renin-angiotensin system and has been implicated in both angiogenesis and pathological vascular growth (Willis *et al.*, 2011). Indeed, an imbalance of the renin-angiotensin system has been associated with the pathogenesis of AAA (Daugherty *et al.*, 2006), and Ang-II-induced AAA formation in apolipoprotein E-deficient mice ($apoE^{-/-}$) shares many characteristic features of the human disease, including chemokine generation, macrophage infiltration and neovascularization (Zhang *et al.*, 2009). Given the high mortality rate associated with AAA in humans, it is important to find new pharmacological approaches to halt its progression.

In this regard, emerging evidence indicates that a single anti-angiogenic drug may not be sufficient to combat the wide array of angiogenic factors produced during AAA and the pathways involved in the angiogenic process. Thus, a potential alternative approach is the use of combinations of already available clinical drugs that exhibit anti-angiogenic activity and whose long-term safety has been proven.

Bexarotene is a RXRα high-affinity synthetic ligand, which is used in the treatment of cutaneous T-cell lymphoma and displays anti-angiogenic activity (Yen and Lamph, 2005). This anticancer drug is currently being tested for the treatment of other metabolic diseases (Lalloyer *et al.*, 2006) and Alzheimer's disease (Cramer *et al.*, 2012). RXRα acts as a transcription factor that on activation binds to gene regulatory DNA sequences and subsequently modulates the transcription of its target genes (Yen and Lamph, 2005). Recently, our group demonstrated that bexarotene exerts anti-inflammatory effects by inhibiting endothelial chemokine release and the expression of adhesion molecules (Sanz *et al.*, 2012). Given that dyslipidaemia is associated with bexarotene treatment in humans, some clinical studies have investigated the ability of statins to counteract this adverse effect (Scarisbrick *et al.*, 2013). In fact, rosuvastatin is the recommended statin for mitigating the hypertriglyceridaemia and/or hypercholesterolaemia caused by bexarotene (Scarisbrick *et al.*, 2013). Its effect seems to lie in the fact that it is not metabolized by CYP3A4 and its minimal metabolic transformation by the CYP2C9 and CYP2C19 isoenzyme pathways (Soran and Durrington, 2008).

Although some of the effects of statins are due to their lipid-lowering properties, it is now well recognized that many of their beneficial actions are related to their pleiotropic effects, including inhibition of inflammation, modulation of endothelial function, anti-angiogenic activities and attenuation of thrombosis (Wang *et al.*, 2010; Babelova *et al.*, 2013; Patterson *et al.*, 2013; Mihos *et al.*, 2014). Furthermore, there is a growing body of evidence suggesting that statins have the

potential to interact with PPARs (Balakumar and Mahadevan, 2012). Although statins are widely used, myopathy and acute renal events are a significant concern surrounding the use of high-potency statin drugs, in particular simvastatin and rosuvastatin (Golomb and Evans, 2008; Dormuth *et al.*, 2013).

Because it is well known that PPARs form permissive RXR heterodimers, which synergistically respond to agonists of RXR and the partner receptor (Plutzky, 2011), the aim of the present study was to examine the combined effect of bexarotene and rosuvastatin on the AAA induced by Ang-II. Herein, we demonstrated for the first time that suboptimal doses of bexarotene and rosuvastatin administered in combination effectively inhibit Ang-II-induced AAA. Most relevantly, this combined therapy significantly prevented Ang-II-induced chemokine expression, monocyte infiltration and neovascularization in apoE$^{-/-}$ mice. Furthermore, we also demonstrated, *in vitro*, that bexarotene plus rosuvastatin at suboptimal concentrations inhibited Ang-II-induced endothelial cell proliferation, migration and tube formation, which correlated with the impaired endothelial release of angiogenic chemokines (C-X-C motif) ligand 1 (CXCL1), (C-C motif) ligand 2 (CCL2), and (C-C motif) ligand 5 (CCL5), as well as VEGF. Activation of RXRα, PPARα or PPARγ, but not PPARβ/δ, was a condition for the anti-angiogenic activity displayed. In addition, inhibition of the phosphatidylinositide 3-kinase (PI3K)–Akt–mammalian target of the rapamycin (mTOR) signalling pathway may account for the synergistic effect produced by this co-treatment.

Methods

Animal studies

The present protocol followed the European Union guidelines for animal care and protection and was approved by the Ethics Review Board of the School of Medicine, University of Valencia. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). C57BL/6 and apoE$^{-/-}$ C57BL/6 male mice were supplied by Charles River Laboratories (Chatillon-sur-Chalaronne, France). The total number of animals used in these studies was $n = 95$. Mice were kept in specific pathogen-free conditions, at a constant temperature of 22 ± 2°C, humidity 60–65% with free access to food and water, and subjected to a 12 h dark/light cycle (lights on at 0800 h).

AAA in apoE$^{-/-}$ mice

Experimental protocol. At 8 weeks of age, Alzet osmotic minipumps (Model 2004) were implanted into apoE$^{-/-}$ C57BL/6 or C57BL/6 wild-type mice in order to administer saline (n = 7–8) or Ang-II (n = 7–10) s.c. at a dose of 500 ng·kg^{-1}·min^{-1} for 28 days, during which mice were fed a Western-type diet (0.15% cholesterol with 42% fat calories; Ssniff, Soest, Germany) to induce AAA. Another group of Ang-II-infused mice received bexarotene (10 mg·kg^{-1}·day^{-1} by oral gavage, n = 6–7), rosuvastatin (10 mg·kg^{-1}·day^{-1} delivered by the osmotic minipumps, n = 6–7) or the combination of both drugs (n = 7–8). The dose of bexarotene administered has previously been found not to inhibit acute TNFα-induced responses *in vivo* (Sanz *et al.*, 2012), whereas rosuvastatin was administered at doses that, when given p.o. in drinking water, did not inhibit *in vivo* vascular inflammation or AAA formation (Wang *et al.*, 2011). Because the s.c. administration of the statin using an osmotic minipump allows 100% drug bioavailability and its p.o. administration results in 34.5% bioavailability in mice (Peng *et al.*, 2009), we chose the former route to ensure a full dosage effect. At the end of the experimental protocol, animals were humanely killed by an overdose of anaesthetic. Their aortas were then harvested and the outer diameter was measured using the imaging processing ImageJ software (NIH Image, Bethesda, MD, USA). Measurement of rosuvastatin plasma levels, blood pressure and lipid profile were also performed as described in Supporting Information.

Immunohistochemistry. Macrophage infiltration and microvessel formation in the aortas were measured as previously described (Zhang *et al.*, 2009). Further details are found in Supporting Information.

RT-PCR. RNA was extracted from the aortas by homogenization and converted to cDNA by standard methods. Further details are found in Supporting Information.

Aortic ring assay. Aortic ring assays were performed as previously described (Piqueras *et al.*, 2007). Details are described in Supporting Information.

Murine Matrigel plug assay. The Matrigel plug assay was performed in 8-week-old C57BL/6 mice as previously described (Piqueras *et al.*, 2007). Details are described in Supporting Information.

Endothelial cell differentiation assay. HUVECs were seeded on growth factor-depleted Matrigel (BD Biosciences, San Jose, CA, USA) as previously described (Piqueras *et al.*, 2007). Further details are found in Supporting Information.

Bromodeoxyuridine (BrdU) proliferation assay. Proliferation assays were performed by BrdU incorporation. Further details are found in Supporting Information.

Wound-healing migration assay. Endothelial migration assays were performed as described in Supporting Information.

Western immunoblotting. Western blot analysis was performed as previously described (Sanz *et al.*, 2012). RXRα, PPARα, PPARβ/δ and PPARγ were determined. Additionally, Akt, mTOR and p70S6K1 phosphorylation was detected by Western blot. Further details are found in Supporting Information.

Immunoprecipitation. Cell extracts were prepared as previously described (Sanz *et al.*, 2012). Protein (~200 μg) from cell extracts was incubated with 5 μg of the antibody against PPARα or PPARγ. Immunocomplexes were precipitated using anti-rabbit IgG beads (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Western blot was performed with an antibody against human RXRα.

Chemokines and VEGF detection. CXCL1, CCL2, CCL5 and VEGF were measured in HUVEC culture supernatants using

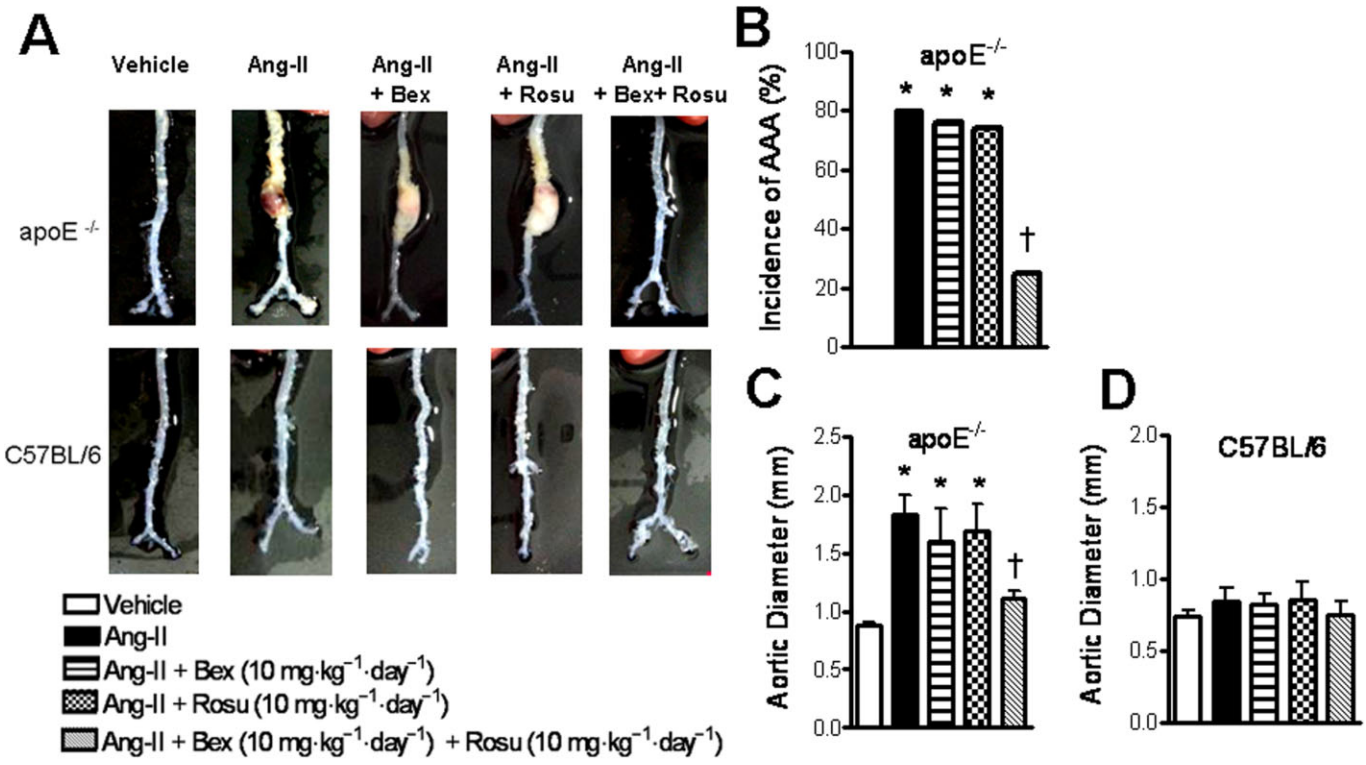

Figure 1

Effects of bexarotene (Bex, 10 mg·kg^{-1}·day^{-1}) in combination with rosuvastatin (Rosu, 10 mg·kg^{-1}·day^{-1}) on Ang-II-induced AAA formation in apoE$^{-/-}$ and C57BL/6 mice. (A) Representative aortas from apoE$^{-/-}$ and C57BL/6 mice. The gross appearances of the aorta were photographed digitally and the maximal external diameter of the suprarenal aorta was measured. A definition of increase in outer diameter of >50% indicated the development of aortic aneurysm. (B) Incidence of AAA in apoE$^{-/-}$ mice. Results are expressed as a percentage. *P < 0.05 versus vehicle-infused mice; †P < 0.05 versus Ang-II-infused animals. (C) Aortic diameter (mm) in apoE$^{-/-}$ and in (D) C57BL/6 mice. Results are the mean ± SEM (n = 6–10 animals per group). *P < 0.05 versus vehicle-infused mice; †P < 0.05 versus Ang-II-infused animals.

Ab pairs from R&D Systems (Abingdon, UK). Further details are found in Supporting Information.

Statistical analysis. Differences between multiple groups were analysed with one-way ANOVA with Bonferroni's procedure for *post hoc* analysis and differences between two groups were determined with Student's *t*-test. Fisher's exact test was used to determine the differences between groups in AAA incidence. Results are expressed as mean ± SEM of the number of independent experiments performed. Data were considered statistically significant when P was <0.05. Data were analyzed using the GraphPad software (GraphPad Prism 4, Inc, La Jolla, CA, USA).

Results

Simultaneous administration of bexarotene and rosuvastatin decreases Ang-II-induced AAA formation, monocyte infiltration, chemokine expression and neovascularization in the aneurysmal tissue

To evaluate the possible effect of bexarotene and/or rosuvastatin on aneurysm formation, apoE$^{-/-}$ and C57BL/6 mice were infused for 28 days with Ang-II and subjected to a Western-type diet. As shown in Figure 1, continuous infusion of apoE$^{-/-}$ mice with Ang-II induced the development of AAA. In contrast, no AAAs were observed in C57BL/6 mice infused with the peptide (Figure 1A) as demonstrated previously (Manning *et al.*, 2002). The infusion of Ang-II resulted in AAA incidence of 80% in apoE$^{-/-}$ mice. However, combined therapy with bexarotene (10 mg·kg^{-1}·day^{-1}) plus rosuvastatin (10 mg·kg^{-1}·day^{-1}) reduced AAA incidence to 25% (Figure 1B). Similarly, while no changes in aortic diameter were detected in C57BL/6 wild-type mice infused with Ang-II, a significant increase in suprarenal aortic diameter was found in apoE$^{-/-}$ mice chronically stimulated with Ang-II (P < 0.05), which in the latter was significantly reduced by the combined treatment with bexarotene and rosuvastatin (P < 0.05) (Figure 1C).

Additionally, an abundant infiltration of Mac-3^{+} macrophages was detected in the media and adventitia of the aortic aneurysms from untreated Ang-II-infused mice (Figure 2). This inflammatory infiltrate was only significantly impaired in those mice subjected to the combined treatment (Figure 2A and B, P = 0.033).

While CD31^{+} microvessels were almost undetectable in the cross-sections of the aortas from control mice, a large number of capillary vessels was observed in untreated

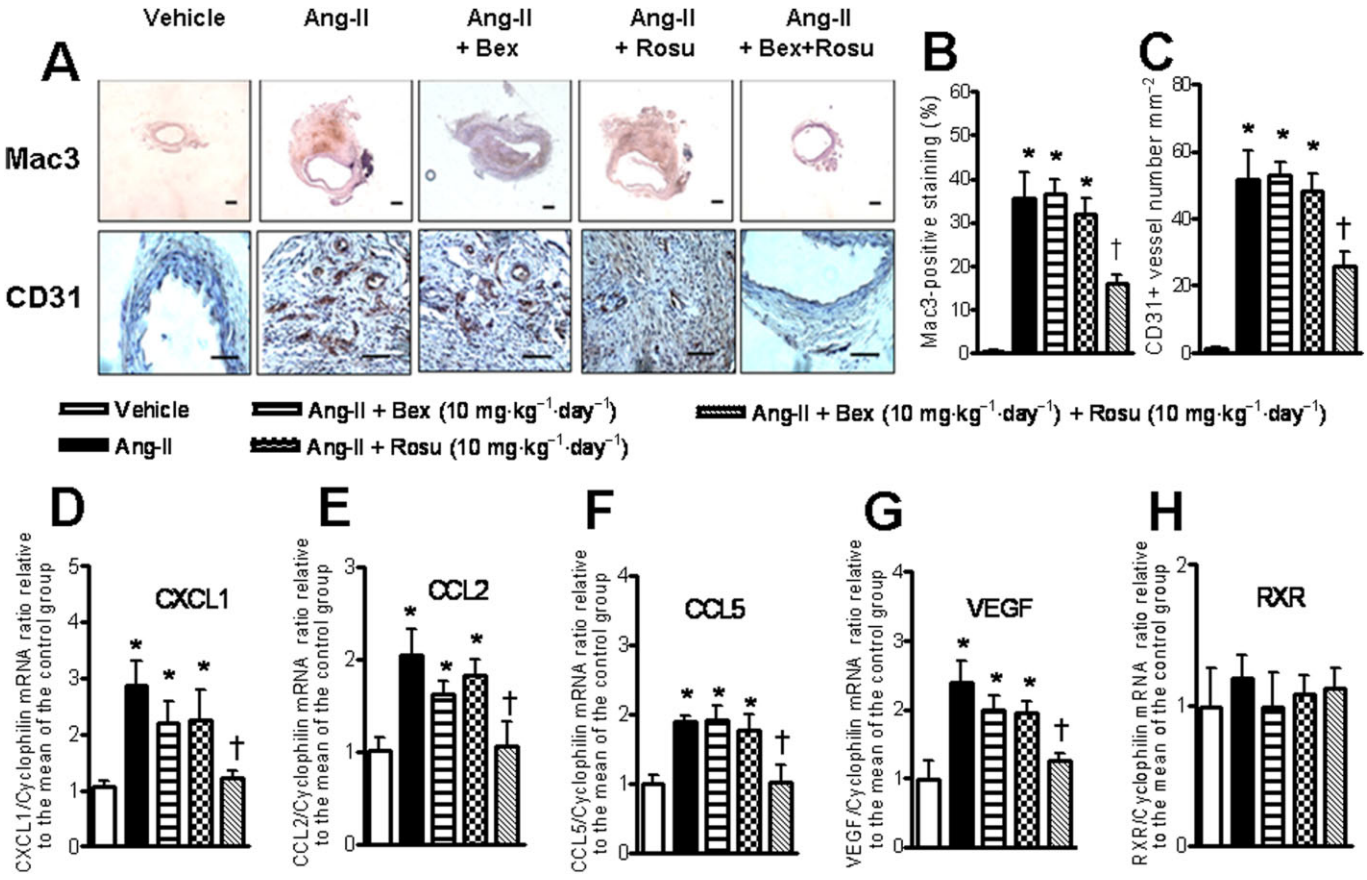

Figure 2

Bexarotene (Bex, 10 mg·kg^{-1}·day^{-1}) in combination with rosuvastatin (Rosu, 10 mg·kg^{-1}·day^{-1}) reduced macrophage infiltration, neovascularization and inflammation in the Ang-II-induced AAA mouse model. (A) Representative photomicrographs of macrophage staining (scale bar, 200 μm) and CD31+ microvessels (scale bar, 50 μm) in aortic cross sections. Bar graphs represent (B) area of Mac-3 positive staining and (C) CD31+ microvessels mm^{-2} in AAA. Results are the mean ± SEM (n = 6 animals per group). *P < 0.05 versus vehicle-infused mice; †P < 0.05 versus Ang-II-infused animals. In the AAA, gene expression of (D) CXCL1, (E) CCL2, (F) CCL5, (G) VEGF and (H) RXR was analysed by real-time RT-PCR. Results are the mean ± SEM of the ratio between each gene and *cyclophilin* gene expression and are referred to mean of the vehicle-treated group set at 1.0 (n = 6 animals per group). *P < 0.05 versus vehicle-infused mice; †P < 0.05 versus Ang-II-infused animals.

Ang-II-stimulated aortas. Interestingly, mice treated simultaneously with bexarotene and rosuvastatin showed a marked decrease in capillary formation (Figure 2A and C, P = 0.030). Moreover, the increased mRNA expression of pro-angiogenic chemokines (CXCL1, CCL2 and CCL5) and VEGF in the aortas from untreated Ang-II-infused mice was markedly reduced by the combination therapy (Figure 2D–G, P < 0.05). Furthermore, no changes in RXRα receptor mRNA expression were found in the AAA induced by Ang-II compared with vehicle-infused controls (Figure 2H, P > 0.05). Moreover, RXRα expression was unaffected by the administration of either bexarotene, rosuvastatin alone or both drugs together (P > 0.05).

We would like to point out that s.c. administration of 10 mg·kg^{-1}·day^{-1} rosuvastatin for 5 days resulted in 108.8 ± 2.0 ng·mL^{-1} circulating levels of the statin determined by LC-electrospray ionization–tandem MS (LC-MS/MS) method (Supplemental Methods; Supporting Information). However, systolic blood pressure was significantly increased in those animals subjected to chronic Ang-II infusion regardless of the strain investigated (Supporting Information Table S1). In apoE$^{-/-}$ but not in C57BL/6 mice, significant increases in total plasma and non-HDL (high-density lipoprotein) cholesterol were found in those individuals chronically infused with Ang-II and fed a Western diet. In contrast, triglycerides and HDL cholesterol concentrations were not affected (Supporting Information Table S1). Finally, chronic administration of bexarotene and/or rosuvastatin did not provoke any changes in the systolic blood pressure and lipid profile of apoE$^{-/-}$ mice chronically stimulated with Ang-II and subjected to a Western diet for 28 days (Supporting Information Table S1).

Bexarotene plus rosuvastatin attenuates Akt/mTOR/p70S6K1 phosphorylation in Ang-II-induced AAA

Because previous studies have observed that the Akt/mTOR/p70S6K1 pathway can also be involved in AAA formation (Lawrence *et al.*, 2004), we next determined the effect of combined bexarotene and rosuvastatin administration on

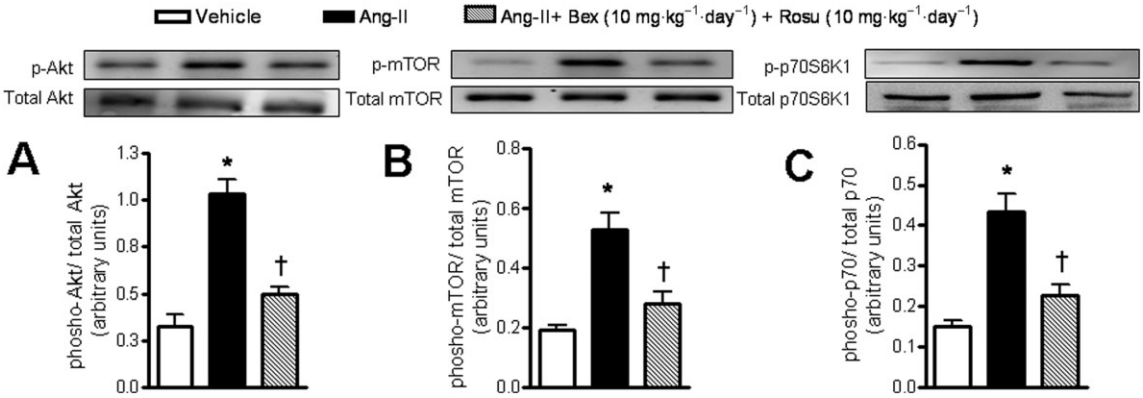

Figure 3

The combined administration of bexarotene (Bex, 10 mg·kg^{-1}·day^{-1}) and rosuvastatin (Rosu, 10 mg·kg^{-1}·day^{-1}) decreased Ang-II-induced phosphorylation of Akt, mTOR and p70S6K1 in the aorta tissue of apoE$^{-/-}$ mice. Bar graphs show protein expression ratios of (A) phospho-Akt/total Akt, (B) phospho-mTOR/total mTOR and (C) phospho-p70S6K1/total p70S6K1 determined by Western blotting of the aneurysm wall tissue. Results are the mean ± SEM (n = 6 animals per group). Representative Western blot gels are also shown. *P < 0.05 versus vehicle-infused mice; †P < 0.05 versus Ang-II-infused animals.

Akt/mTOR/p70S6K1 activation in homogenates of aneurysmal tissue. Western blot analysis of the aorta revealed increases in Akt (Figure 3A), mTOR (Figure 3B) and p70S6K1 (Figure 3C) phosphorylation in apoE$^{-/-}$ mice subjected to Ang-II infusion. Interestingly, the combination therapy attenuated Ang-II-induced Akt, mTOR and p70S6K1 activation (Figure 3, P < 0.05).

A combination of bexarotene and rosuvastatin at suboptimal concentrations reduces endothelial tube formation, proliferation and migration induced by Ang-II

To further evaluate the effect of the combined bexarotene and rosuvastatin treatment on angiogenesis, HUVECs were stimulated with 1 μM Ang-II for 24 h. Bexarotene (0.3–10 μM) and rosuvastatin (1–10 μM) inhibited, in a concentration-dependent manner, the tube-like structures induced by the peptide hormone (Figure 4A and B). Interestingly, while suboptimal concentrations of bexarotene (0.3 μM) or rosuvastatin (3 μM) were unable to inhibit Ang-II-induced tube formation, significant reductions in this parameter were achieved when the two compounds were administered together (Figure 4C and D, P < 0.001). As expected, pretreatment of the cells with 100 μM EXP3174, an AT_1 Ang-II receptor antagonist, inhibited Ang-II-induced tubulogenesis (Figure 4C).

The proliferation and migration of endothelial cells are essential steps in angiogenesis (Piqueras *et al.*, 2007), and we found that pretreatment of these cells with bexarotene (0.3 μM) plus rosuvastatin (3 μM), but not with either agent alone, decreased Ang-II-induced HUVEC proliferation by 52% (Figure 5A, P = 0.001). Similarly, a significant reduction in the migratory potential of Ang-II-stimulated HUVECs was observed when bexarotene and rosuvastatin were both present (25% inhibition, Figure 5B and C, P = 0.023).

Bexarotene in combination with rosuvastatin at suboptimal concentrations inhibits endothelial cell sprouting and Matrigel vascularization in mice

In order to confirm the synergistic anti-angiogenic activity exerted by combined bexarotene/rosuvastatin treatment, we next tested the activity of both drugs in the *ex vivo* murine aortic ring assay and in the *in vivo* Matrigel assay. In the *ex vivo* murine aortic ring assay, 8 days of stimulation with Ang-II (1 μM) resulted in increased endothelial cell sprouting versus vehicle-exposed aortic segments, an effect mediated by Ang-II activation of AT_1 receptors (Figure 6A). In agreement with our previous observations, incubation with both drugs markedly reduced Ang-II-induced angiogenesis (Figure 6A, P < 0.001).

In contrast, in the *in vivo* Matrigel assay, Ang-II-induced increases in Hb content were blunted by EXP3174 (Figure 6B). Interestingly, the combined treatment with both bexarotene and rosuvastatin induced a significant decrease in the Matrigel plug Hb content compared with that in plugs containing only Ang-II (Figure 6B, P < 0.001).

Inhibition of Ang-II-induced endothelial angiogenic chemokines and VEGF production by a combination of bexarotene plus rosuvastatin at suboptimal concentrations

Significant increases in CXCL1, CCL2 and CCL5 levels were detected in the supernatants of Ang-II-stimulated HUVEC, mediated through activation of AT_1 receptors (Figure 7A–C). Notably, the combination of bexarotene (0.3 μM) with rosuvastatin (3 μM) synergistically reduced the Ang-II-induced increase in CXCL1, CCL2 and CCL5 levels by 64, 43 and 41% respectively (Figure 7A–C, P < 0.05). To determine the potential contribution of these chemokines to the angiogenic activity of Ang-II, different chemokine receptors were selectively blocked. As shown in Figure 7D, the CXCR2 antagonist that

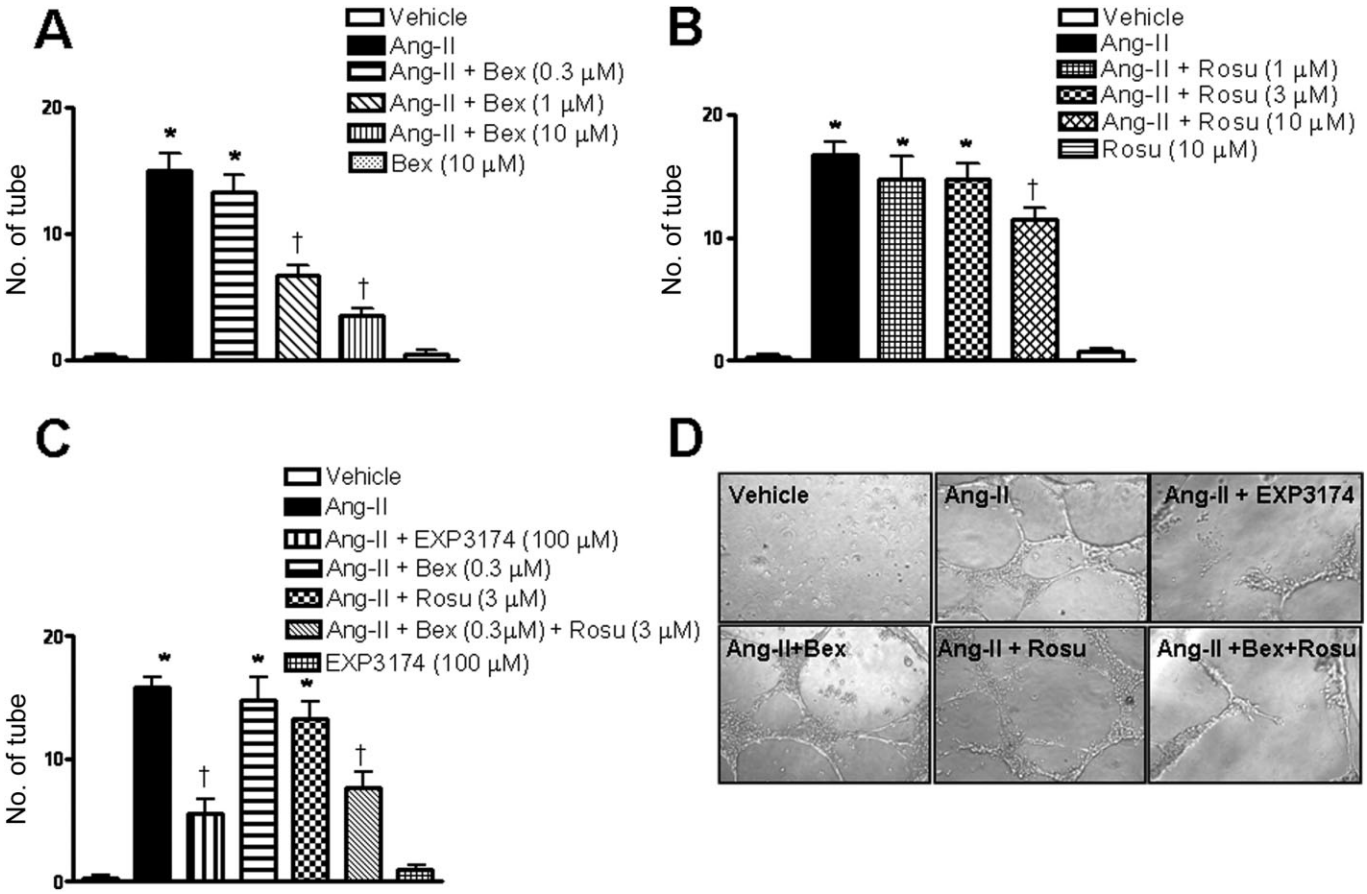

Figure 4

The combination of bexarotene and rosuvastatin at suboptimal concentrations decreased Ang-II-induced tube formation. Endothelial differentiation assay was performed on Matrigel. HUVECs were incubated with vehicle (0.01 % DMSO), bexarotene (Bex, 0.3–10 μM) (A) or rosuvastatin (Rosu, 1–10 μM) (B) 24 h before Ang-II stimulation (1 μM, 24 h). In other experiments, HUVECs were pretreated with EXP3174 (100 μM, 1 h) or with bexarotene (0.3 μM), rosuvastatin (3 μM) or both for 24 h and then stimulated with Ang-II (24 h) (C). Results are the mean ± SEM of the number of tube-like structures in five low-magnification (×100) fields ($n = 6$ independent experiments). *$P < 0.01$ versus vehicle; †$P < 0.05$ versus Ang-II. (D) Representative images of the endothelial differentiation assays.

blocks most of the action of the glutamic acid-leucine-arginine or ELR$^+$-CXC chemokines only partially reduced the tube formation caused by Ang-II (21% inhibition, $P = 0.033$). Interestingly, CCR2 blockade decreased Ang-II-induced capillary-like structure formation by 45% (Figure 7E, $P = 0.042$). Similarly, tubulogenesis induced by Ang-II was markedly diminished when CCR1, CCR3 and CCR5 receptors were simultaneously blocked (51% inhibition, Figure 7E, $P = 0.020$).

Ang-II can also cause the generation and release of the angiogenic factor VEGF from endothelial cells (Willis *et al.*, 2011). Firstly, we found that 1 h preincubation with EXP3174 (100 μM) reduced the VEGF release evoked by Ang-II (Supporting Information Fig. S1A, $P < 0.001$). Secondly, bexarotene plus rosuvastatin diminished Ang-II-induced VEGF release by 40% (Supporting Information Fig. S1A, $P < 0.001$). Finally, pre-incubation with a VEGFR-1/2 antagonist drastically decreased Ang-II-induced tubulogenesis (Supporting Information Fig. S1B, $P < 0.001$).

RXRα and its heterodimer partners PPARα and PPARγ are involved in the anti-angiogenic activity exerted by the combination of suboptimal concentrations of bexarotene plus rosuvastatin

To achieve further insights into the underlying mechanism, we first investigated the potential involvement of RXRα. Forty-eight hours post transfection with a RXRα-specific siRNA, HUVEC showed a >75% reduction in RXRα protein levels compared with control siRNA-transfected cells (Supporting Information Fig. S2A, $P < 0.001$). Of importance, RXRα-specific siRNA abolished the suppressive effects of bexarotene plus rosuvastatin on the tubulogenesis induced by Ang-II (Figure 8A and E, $P < 0.050$).

RXRα can dimerize with other nuclear hormone receptors that are also potent regulators of angiogenesis, such as PPARs (Xin *et al.*, 1999; Varet *et al.*, 2003; Piqueras *et al.*, 2007). Therefore, we sought to explore the potential involvement of

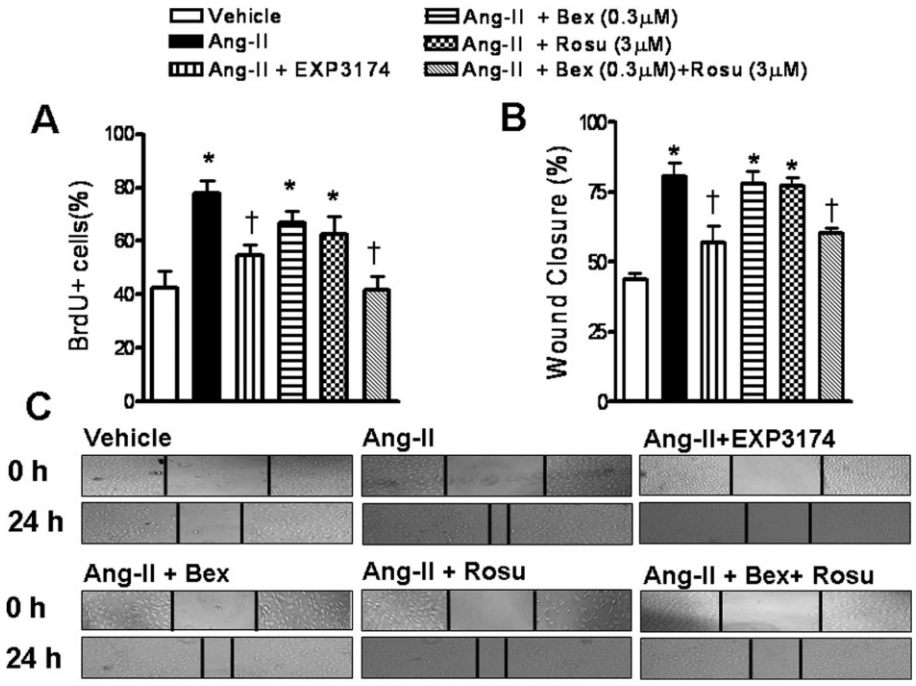

Figure 5

The combination of bexarotene (Bex, 0.3 μM) and rosuvastatin (Rosu, 3 μM) at suboptimal concentrations decreased Ang-II-induced endothelial cell proliferation and migration. (A) Percentage of proliferating endothelial cells was analysed by BrdU incorporation. *$P < 0.05$ versus vehicle; †$P < 0.05$ versus Ang-II. Results are the mean ± SEM ($n = 5$). (B) Bar graph represents percentage of wound closure with the different treatments. The degree of wound closure was measured as the percentage of the area covered by migrating cells in the initial wound. Results are the mean ± SEM ($n = 5$). (C) Representative photomicrographs of wounds ($t = 0$ and $t = 24$ h); black lines highlight the linear scratch/wound.

PPARs in the responses produced by the drug combination. Again, a siRNA approach was employed (Supporting Information Fig. S2). While the inhibitory effects of the bexarotene/rosuvastatin combination on Ang-II-induced capillary formation were reversed in PPARα and PPARγ siRNA-transfected cells (Figure 8B and D, $P < 0.05$), PPARβ/δ knockdown did not affect these responses (Figure 8C). Additionally, immunoprecipitation assays revealed that RXRα/PPARα and RXRα/PPARγ interactions were enhanced when endothelial cells were pretreated with the drug combination (Figure 8F).

Bexarotene in combination with rosuvastatin at suboptimal concentrations inhibits the activation of the Akt/mTOR/P70S6K1 signalling pathway induced by Ang-II

Because the PI3K/Akt/mTOR pathway seems to play a role in Ang-II-induced AAA formation, we next examined its potential involvement in the angiogenic response provoked by Ang-II in HUVEC. The 15 min challenge with 1 μM Ang-II triggered a marked phosphorylation of Akt, mTOR and p70S6K1 through interaction with its AT_1 receptor (Figure 9). Pre-incubation of the cells with bexarotene (0.3 μM) plus rosuvastatin (3 μM) decreased Ang-II-induced phosphorylation of the different members of this signalling pathway (Figure 9A–C, $P < 0.05$). Moreover, this effect was replicated when the cells were pre-incubated with LY294002 (10 μM), a selective PI3K inhibitor (Figure 9D–F). Furthermore, the inhibition of PI3K decreased the production of pro-angiogenic chemokines CXCL1, CCL2 and CCL5, and VEGF and the tubulogenesis exerted by Ang-II (Supporting Information, Figure 3A–E, $P < 0.05$).

Discussion and conclusions

The search for new strategies to prevent and/or slow down the progression of angiogenesis associated with cardiovascular diseases such as AAA is critical in order to reduce the occurrence of severe complications derived from new vessel formation. This is the first study to report a positive effect of bexarotene in combination with rosuvastatin on the suppression of Ang-II-induced AAA formation in apoE$^{-/-}$ mice. This combined therapy inhibited the proliferation, migration and vessel network formation induced by Ang-II in *in vitro* and *in vivo* models of angiogenesis.

Experimental and clinical evidence endorses the relevance of the renin-angiotensin system in AAA pathogenesis (Daugherty *et al.*, 2006; Zhang *et al.*, 2009), where increased adventitial neocapillary formation is a key feature (Nishibe *et al.*, 2010). In our study, bexarotene and rosuvastatin were co-administered at doses that did not exert any significant effects on vascular inflammation *in vivo* (Wang *et al.*, 2011; Sanz *et al.*, 2012). However, we observed that chronic co-administration of bexarotene with rosuvastatin synergistically reduced aneurysm formation. This effect was accompanied by decreased macrophage and vessel numbers within the

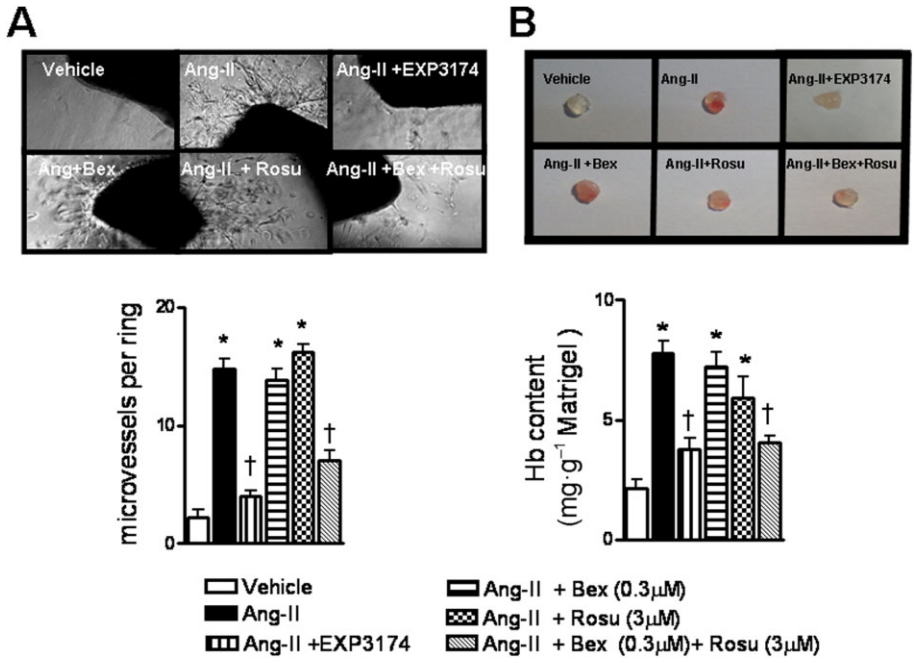

Figure 6

The combination of suboptimal concentrations of bexarotene (Bex, 0.3 μM) and rosuvastatin (Rosu, 3 μM) decreased endothelial cell outgrowth in the *ex vivo* murine aortic ring assay and reduced vascularization in the *in vivo* Matrigel plug assay. (A) Figure shows quantitative analysis of microvessel sprouting after 8 days with treatment with EXP3174 (100 μM) or with bexarotene (0.3 μM), rosuvastatin (3 μM) or both and stimulated with Ang-II (1 μM). The number of microvessels per aortic ring was counted, three rings per treatment group were analysed for each mouse. Results are the mean ± SEM (n = 5 animals per group). *P < 0.01 versus vehicle; †P < 0.05 versus Ang-II. (B) Figure shows Hb content in the Matrigel plugs. Results are the mean ± SEM (n = 5). *P < 0.01 versus vehicle plugs; †P < 0.05 versus Ang-II plugs.

lesions, events not detected in untreated animals or in those subjected to a single treatment. Additionally, in our study, a clear down-regulation of aortic CXCL1, CCL2 and CCL5 and VEGF mRNA expression was detected in those animals simultaneously administered with the drug combination. In this regard, the CCL2/CCR2 axis has been shown to be involved in Ang-II-induced AAA formation (Izhak *et al.*, 2012), and dual blockade of CCR1 and CCR5 markedly limited the accumulation of monocytes within the experimental aortic aneurysm lesion, leading to the inhibition of matrix-degrading protease release, angiogenesis and preservation of the aortic structure (Middleton *et al.*, 2007; Suffee *et al.*, 2012). Whether these events are linked or not to IL-6 production as found in other human and animal studies (Wojcik *et al.*, 2011; Wang *et al.*, 2013) remains to be determined. Moreover, it is also likely that the decreased VEGF generation could be attributed to the reduced monocyte infiltration elicited by the drug combination because this angiogenic mediator can be produced by inflammatory monocytes/macrophages recruited during aortic aneurysm development (Nishibe *et al.*, 2010) in addition to endothelial cells.

In addition, increased expression of phospho-Akt and phospho-mTOR in the aortic wall of the AAA induced by Ang-II was markedly attenuated by co-treatment of the animals with the RXR ligand and the statin. In this regard, genetic mutations in the upstream negative regulators of mTOR have been associated with aorta aneurysm formation in experimental animal models and in humans (Cao *et al.*, 2010), and treatment with mTOR inhibitors has also been found to prevent AAA formation (Lawrence *et al.*, 2004). Thus, the angiopreventive effects reported in the current study suggest that, *in vivo*, inhibition of the Akt/mTOR pathway seems to play a key role in AAA formation probably limiting the release of inflammatory/pro-angiogenic molecules.

Recent evidence strongly indicates that inflammation and angiogenesis are interconnected (Mantovani *et al.*, 2008). Indeed, newly formed blood vessels enable the continuous recruitment of inflammatory cells which release a variety of pro-angiogenic chemokines and growth factors such as VEGF, thereby amplifying the angiogenic process (Mantovani *et al.*, 2008). The pro-angiogenic effects of Ang-II through its interaction with its AT_1 receptor are well established (Tamarat *et al.*, 2002), and it is known that stimulation of vascular endothelial cells with Ang-II results in the production of a wide array of leukocyte-recruiting chemokines and VEGF (Tamarat *et al.*, 2002; Nabah *et al.*, 2004; Mateo *et al.*, 2006; Willis *et al.*, 2011). Indeed, a subset of these chemotactic cytokines has been implicated in angiogenesis. In this context, ELR^+-CXC chemokines such as CXCL1 (GROα), which act mainly on the CXCR2, are primarily neutrophil chemoattractants, but also display angiogenic activity (Bechara *et al.*, 2007). However, the previously mentioned CC chemokines such as CCL2 and CCL5 have recently been reported to be involved in neovascularization (Izhak *et al.*, 2012; Suffee *et al.*, 2012). In our study, we demonstrated that while the selective block of the CXCR2 resulted in a moderate decrease in Ang-II-induced morphogenesis, the blockade of

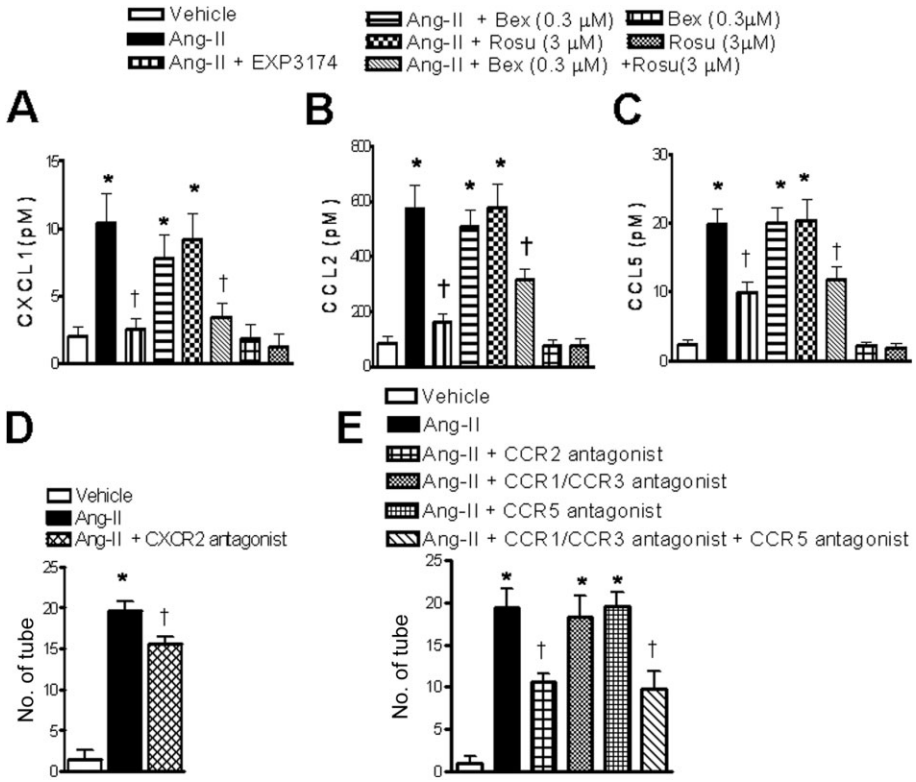

Figure 7

Inhibition of (A) CXCL1, (B) CCL2 and (C) CCL5 synthesis in Ang-II-stimulated HUVEC by pretreatment with a combination of suboptimal concentrations of bexarotene (Bex, 0.3 μM) and rosuvastatin (Rosu, 3 μM). Results are the mean ± SEM (n = 5–6). In other experiments, HUVECs were seeded on Matrigel and pretreated with a CXCR2 antagonist (SB225002, 100 nM) (D), a CCR2 antagonist (BMSCCR222, 10 μM), the dual CCR1/CCR3 antagonist (UCB35625, 100 nM) and/or the CCR5 antagonist (DAPTA, 10 nM) (E), 1 h before Ang-II stimulation (24 h). Results are the mean ± SEM (n = 5). *P < 0.05 versus vehicle; †P < 0.01 versus Ang-II.

CCR2 and simultaneous antagonizing of CCR1, CCR3 and CCR5, or VEGFR1/2 drastically reduced the angiogenic response elicited by Ang-II. Thus, CCL2, CCL5 and VEGF seem to be key molecules in the angiogenic activity elicited by this peptide hormone.

On the other hand, RXR agonists (Yen *et al.*, 2006) and statins (Park *et al.*, 2002; Wang *et al.*, 2010) have each been shown to suppress angiogenesis in *in vivo* and *in vitro* models, mainly through VEGF inhibition. In this context, previous data generated by our group have revealed that RXR agonists such as bexarotene or 9-cis retinoic acid reduce the endothelial release of CXCL1 and CCL2 in TNFα-stimulated cells, an effect that is not produced in cells lacking RXRα (Sanz *et al.*, 2012). Furthermore, the production of MCP-1, IL-1, IL-6 and IL-8 among other cytokines or chemokines by endothelial or different cell types is known to be inhibited by statins (Veillard *et al.*, 2006; Mayer *et al.*, 2007; Jougasaki *et al.*, 2010; Girardi *et al.*, 2011; Patterson *et al.*, 2013; Xiao *et al.*, 2013). Therefore, the anti-angiogenic activity displayed by the bexarotene/rosuvastatin combination seems to be the consequence of the inhibition of the generation and release of CC angiogenic chemokines, CCL2 and CCL5, and the inhibition of Ang-II-evoked VEGF production. Because three different RXR isotypes have been described – namely RXRα, RXRβ and RXRγ (Szanto *et al.*, 2004) – we silenced RXRα endothelial expression in an attempt to address whether this isotype was responsible for the observed effects. The lack of RXRα expression blunted the inhibition of Ang-II-induced neovascularization exerted by bexarotene and rosuvastatin co-treatment, indicating that the inhibition of angiogenesis depends on the activation of this nuclear receptor. Moreover, RXRs are common heterodimerization partners of other nuclear receptors, and it is well known that PPARs form permissive RXR heterodimers (Plutzky, 2011). Alternatively, statins activate both PPARα and PPARγ in endothelial cells (Balakumar and Mahadevan, 2012) and PPARα activation inhibits human dermal endothelial cell proliferation, migration and tube formation *in vitro* through cytoskeleton disruption and inhibition of the critical endothelial cell survival factor Akt (Varet *et al.*, 2003). Similarly, PPARγ agonists also were effective at inhibiting angiogenesis in different experimental models (Xin *et al.*, 1999). In contrast to PPARα and PPARγ, activation of PPARβ resulted in the induction of angiogenesis *in vitro* and *in vivo* (Piqueras *et al.*, 2007). Given all of this evidence, we have additionally demonstrated that knockdown of PPARα or PPARγ, but not of PPARβ, reversed the reduction in Ang-II-induced tube formation produced by the combination of suboptimal concentrations of bexarotene and rosuvastatin. Furthermore, our results also indicate that RXRα requires both PPARα and PPARγ activation to exert its anti-angiogenic

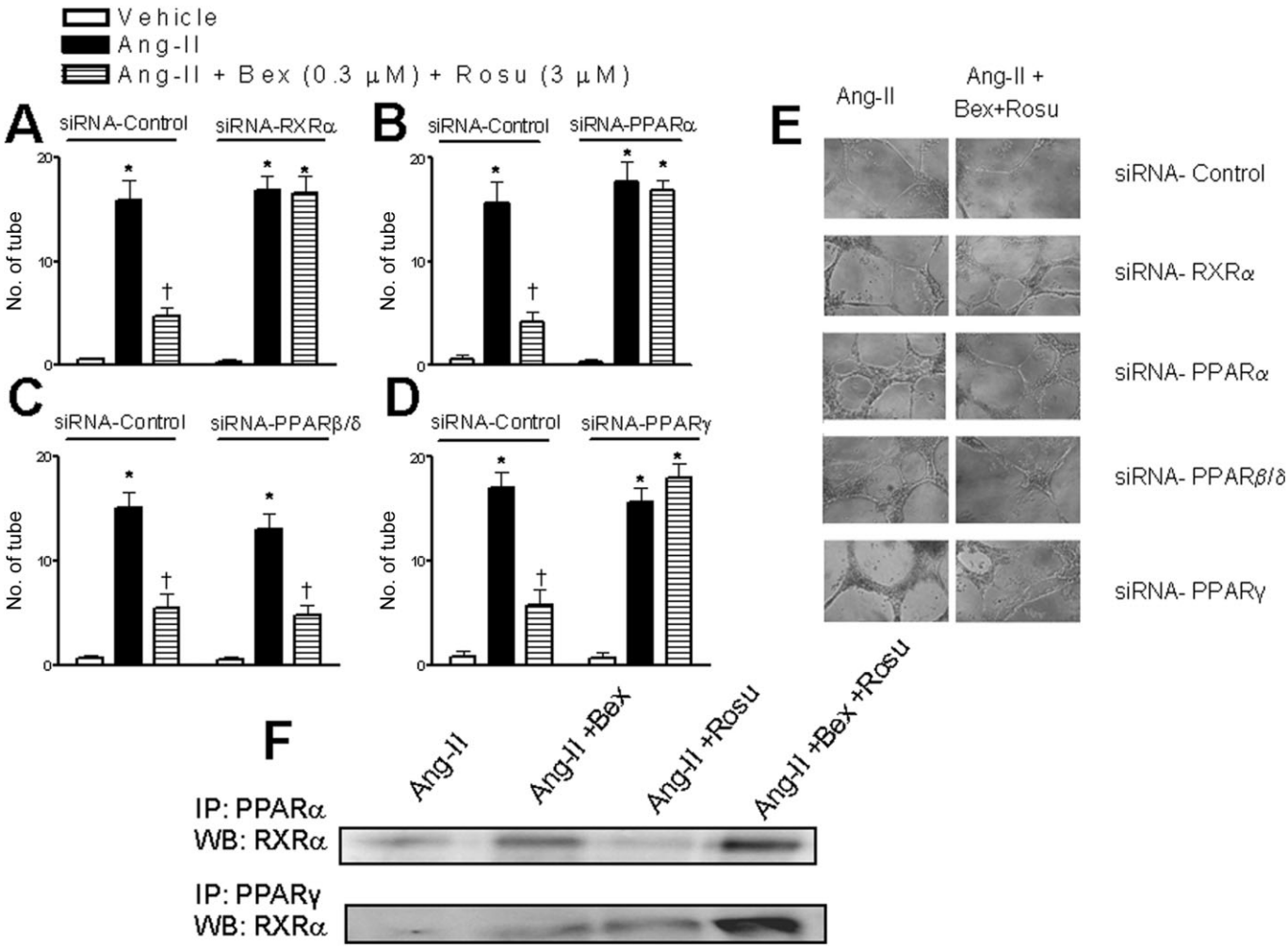

Figure 8

Knockdown of RXRα, PPARγ or PPARα but not PPARβ by small interference RNA attenuated the inhibitory effect of bexarotene plus rosuvastatin in combination on Ang-II-induced endothelial cell tube formation. HUVECs were transfected with control, (A) RXRα, (B) PPARα, (C) PPARβ/δ or (D) PPARγ-specific siRNA. Forty-eight hours post transfection, the cells were pretreated with bexarotene (Bex, 0.3 μM) and/or rosuvastatin (Rosu, 3 μM) for 24 h. Then the cells were stimulated with Ang-II (1 μM, 24 h). The number of tube-like structures determined in five low-magnification (×100) fields was quantified. Results are the mean ± SEM of $n = 5–6$ independent experiments. *$P < 0.05$ relative to the vehicle group. †$P < 0.05$ relative to the Ang-II-stimulated cells. (E) Panel shows representative photomicrographs of the endothelial differentiation assay. (F) RXRα/PPARγ or RXRα/PPARα interaction was assessed by immunoprecipitation of PPARγ or PPARα and subsequent Western blotting for RXRα. Blots are representative of $n = 6$ independent experiments.

activity. In this regard, while our findings are in accordance with other studies in which bexarotene did not inhibit tumour invasion and angiogenesis in the absence of PPARγ (Yen *et al.*, 2006), the interaction of RXRα/PPARα as a requirement for combined drug activity has not previously been demonstrated. In the light of all this evidence, it is conceivable that the synergism between the RXR ligand and the statin during angiogenesis inhibition was the consequence of RXRα/PPARγ and RXRα/PPARα heterodimerization.

Given that the PI3K/Akt/mTOR pathway seems to be involved in Ang-II-induced AAA, we next investigated its role in endothelial cell proliferation induced by Ang-II in human endothelial cells. In fact, phosphorylated mTOR-mediated signalling responses are reported to be important in endothelial cell activation, including the induced expression of adhesion molecules or the generation of chemokines and VEGF (Karnoub and Weinberg, 2008; Wang *et al.*, 2014). Furthermore, activation of the PI3K/Akt/mTOR pathway is involved in Ang-II-induced cardiac and vascular hypertrophy and remodelling (Hafizi *et al.*, 2004; Stuck *et al.*, 2008) and in the inhibition of NO release and vasodilatation by insulin stimulation of endothelial cells (Kim *et al.*, 2012). We now show that combined treatment with bexarotene and rosuvastatin at suboptimal concentrations blocked the phosphorylation of different members of the PI3K/Akt/mTOR/p70S6K signalling pathway in Ang-II-stimulated HUVEC. This effect is probably due to the increased RXRα/PPARα and RXRα/PPARγ interactions elicited by this drug combination. In regard to this, there are

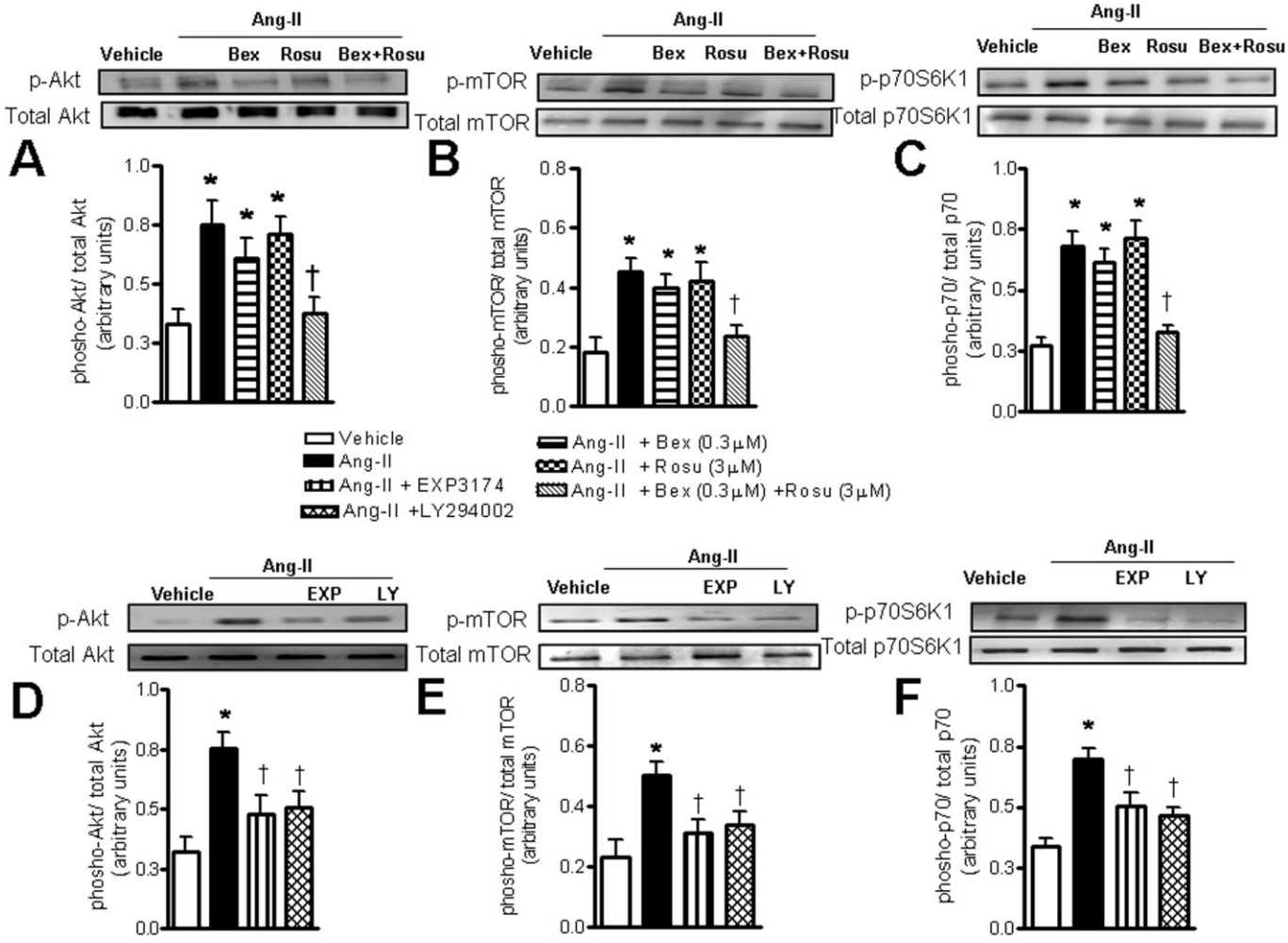

Figure 9

Phosphorylation of Akt, mTOR and p70S6K induced by Ang-II was reduced by pretreatment of the cells with the combination of suboptimal concentrations of bexarotene and rosuvastatin. Cells were pretreated with bexarotene (Bex, 0.3 μM) and/or rosuvastatin (Rosu, 3 μM) for 24 h. After treatments, HUVECs were stimulated with Ang-II (1 μM, 15 min). Bar graphs represent protein expression ratios of (A) phospho-Akt/total Akt, (B) phospho-mTOR/total mTOR and (C) phospho-p70S6K1/total p70S6K1 determined by Western blotting. Results are the mean ± SEM of n = 5 independent experiments. *P < 0.05 versus vehicle; †P < 0.05 versus Ang-II. In another set of experiments, some plates were pretreated with EXP3174 (100 μM) or a PI3K inhibitor (LY294002, 10 μM) 1 h before Ang-II stimulation, and protein expression ratios of (D) phospho-Akt/total Akt, (E) phospho-mTOR/total mTOR and (F) phospho-p70S6K1/total p70S6K1 were determined. Results are the mean ± SEM of n = 5 independent experiments. Representative gels are also shown. *P < 0.05 versus vehicle; †P < 0.05 versus Ang-II.

several lines of evidence that support this hypothesis. Firstly, previous studies have shown that RXRα/PPARγ activation can lead to mTOR and p70S6K pathway inactivation in hepatocytes (Sharvit *et al.*, 2013). Secondly, combined treatment with RXRα and PPARγ agonists also inhibits this signalling pathway (Lee *et al.*, 2006). Thirdly, this pathway can lead to NF-κB activation (Richmond, 2002), resulting in increased production of angiogenic chemokines (Richmond, 2002) and VEGF (Angelo and Kurzrock, 2007; Karar and Maity, 2011), which may account for the counter interplay between inflammation and angiogenesis. In line with this, we have also provided evidence that PI3K inhibition diminished different angiogenic mediator levels released by the peptide hormone.

In conclusion, this is the first study to demonstrate that a combination of suboptimal doses of bexarotene and rosuvastatin results in a potent inhibition of Ang-II-induced AAA. In addition, activation of interactions between RXRα/PPARα and RXRα/PPARγ seem to be required for this inhibitory effect on the PI3K/Akt/mTOR/P70S6K signalling pathway that causes the subsequent impairment of endothelial angiogenic chemokines and VEGF release. Therefore, our results suggest that a therapeutic regimen that combines a RXRα ligand with a statin represents a promising pharmaceutical strategy for the suppression of angiogenesis and prevention of AAA. Furthermore, the use of low doses of both drugs may reduce the appearance of drug-associated side effects, thus providing a safer therapy.

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Authors contributions

L. P. conceived the study and acquisition of data, performed the experiments, analysis and interpretation of the results, and wrote the manuscript. M. J. S. was involved in the conception, hypothesis delineation and design of the study as well as in the manuscript writing. P. E., H. G. N., C. F., M. J. and E. F. performed the *in vitro* assays. A. N. performed the *in vivo* studies, RT-PCR and immunohistochemistry assays.

Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Bexarotene plus rosuvastatin at suboptimal concentrations inhibited VEGF release from Ang-II-stimulated HUVEC.

Figure S2 Gene silencing was performed using either control or RXRα, PPARα, PPARβ/δ or PPARγ-specific siRNA.

Figure S3 Inhibition of PI3K decreases Ang-II-induced chemokine and VEGF production and tube formation.

Table S1 Effect of bexarotene and rosuvastatin on systolic blood pressure and lipid profile in apoE$^{-/-}$ and C57BL6 mice.