β-Arrestin-2 Deficiency Attenuates Abdominal Aortic Aneurysm Formation in Mice

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Abstract

Rationale—Abdominal aortic aneurysms (AAAs) are a chronic inflammatory vascular disease for which pharmacological treatments are not available. A mouse model of AAA formation involves chronic infusion of angiotensin II (AngII) and previous studies indicated a primary role for the angiotensin II type 1a (AT1a) receptor, in AAA formation. β-arrestin-2 (βarr2) is a multifunctional scaffolding protein that binds G-protein coupled receptors such as AT1a, and regulates numerous signaling pathways and pathophysiological processes. However, a role for βarr2 in AngII-induced AAA formation is currently unknown.

Objective—To determine if βarr2 played a role in AngII-induced AAA formation in mice.

Methods and Results—Treatment of βarr2$^{+/+}$ and βarr2$^{-/-}$ mice on the hyperlipidemic ApoE$^{-/-}$ background or normolipidemic C57BL/6 background with AngII for 28 days indicated that βarr2 deficiency significantly attenuated AAA formation. βarr2 deficiency attenuated AngII-induced expression of cyclooxygenase-2 (COX-2), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP1α), and macrophage infiltration. AngII also increased the levels of phosphorylated-extracellular signal-regulated kinase 1/2 (p-ERK1/2) in ApoE$^{-/-}$/βarr2$^{+/+}$ aortas, whereas βarr2 deficiency diminished this increase. Furthermore, inhibition of

ERK1/2 activation with CI1040 (100mg/kg/day) reduced the level of AngII-induced COX-2 expression in ApoE$^{-/-}$/βarr2$^{+/+}$ mice to the level observed in ApoE$^{-/-}$/βarr2$^{-/-}$ mice. AngII treatment also increased matrix metalloproteinase (MMP) expression and disruption of the elastic layer in ApoE$^{-/-}$/βarr2$^{+/+}$ aortas and βarr2-deficiency reduced these effects.

Conclusions—βarr2 contributes to AngII-induced AAA formation in mice by p-ERK1/2-mediated COX-2 induction and increased inflammation. These studies suggest that for the AT1a receptor, G-protein-independent, βarr2-dependent signaling plays a major role in AngII-induced AAA formation.

Keywords

Aneurysm; β-Arrestin-2; cyclooxygenase-2; angiotensin

Introduction

Abdominal aortic aneurysms (AAAs) are a common vascular condition associated with several risk factors including advanced age, male gender, smoking and hypercholesterolemia.[1] AAA formation begins as an abnormal dilation of the aorta, which may gradually expand over a period of years followed by eventual weakening and rupture of the vessel wall, resulting in mortality in about 90% of cases.[2-4] The pathophysiology of AAAs can be roughly categorized into two processes: inflammation and extracellular matrix degeneration.[5] Inflammation is exemplified by the release of inflammatory mediators such as monocyte chemoattractant protein 1 (MCP-1) and interleukin 6 and the resultant infiltration of inflammatory cells, particularly macrophages, into the vessel wall.[6-9] Inflammatory cells are a major source of proteolytic enzymes such as matrix metalloproteinase 2 (MMP2) and MMP9 that are known to disrupt the structural integrity of the vessel wall and degrade the components of the extracellular matrix such as elastin and collagen.[3, 10, 11] This extracellular matrix degeneration and vascular remodeling contribute to the progression and severity of the disease. Currently, there are no pharmacological treatments for AAAs with endovascular or surgical repair being the only options.

A widely used mouse model of AAAs involves chronic infusion of angiotensin II (AngII) and this model displays multiple characteristics of human AAAs and has provided mechanistic insight into the formation and progression of the disease.[12, 13] AngII mediates many of its physiological and pathological effects by activating the G protein coupled receptor (GPCR) AT1 in most species, and the 'a' subtype of AT1 (AT1a) in rodents.[14-17] Genetic ablation of the AT1a receptor in mice or treatment of mice with the AT1 receptor antagonist, losartan, attenuates AngII-induced AAA formation, indicating a critical role for this receptor in AngII-induced AAA pathology.[17, 18]

The traditional signaling pathway employed by the AT1a receptor involves G-protein mediated activation of phospholipase Cβ and the liberation of the second messengers, diacylglycerol and inositol-triphosphate, resulting in downstream signaling events.[16] However, in recent years it has become increasingly evident that AT1a is also involved in G-protein-independent signaling by binding to scaffolding proteins known as β-arrestin-1 (βarr1) and β-arrestin-2 (βarr2).[19, 20] βarr1 and βarr2 are ubiquitously expressed

multifunctional proteins, which were previously thought to be important in termination of GPCR signaling.[19, 20] However, studies over the past decade have demonstrated that βarr1 and βarr2 can mediate G-protein-independent signaling, and contribute to various physiological and pathological conditions.[21-23] The coupling of βarr1 and βarr2 with GPCRs is known to recruit signaling molecules such as the c-Src family kinases, protein kinase B (AKT), phosphotidylinositol 3 kinase, as well as key players in the mitogen activated protein kinase (MAPK) signaling pathway, including p38, c-Jun N terminal kinase 3 and extracellular signal-regulated kinase 1/2 (ERK1/2).[19, 24] The ERK1/2 cascade is the best-studied example of βarr-activated signaling for the AT1a receptor. Studies have shown that βarr1 and βarr2 bind with equal affinity to AT1a, however both isoforms demonstrate differential roles in ERK1/2 signaling, wherein βarr2 is thought to be the primary isoform responsible for ERK1/2 activation.[25]

Cyclooxygenase-2 (COX-2) is an enzyme important in the synthesis of inflammatory mediators such as prostaglandin (PG) E_2, and has been shown to contribute to human AAA formation.[9, 26] Using the AngII-induced AAA model, we previously reported that genetic or pharmacological inactivation of COX-2 attenuated AAA incidence and severity in mice.[8, 27] Furthermore, these studies showed that AngII treatment significantly induced COX-2 expression in the abdominal aorta during the initiation and progression of the disease.[8] These studies also suggested that the increased COX-2 expression in smooth muscle cells may contribute to AAA formation by initiating macrophage infiltration into the aorta via the release of inflammatory chemokines including MCP-1 and macrophage inflammatory protein 1α (MIP1α).[8] Additional studies have shown that the genetic deficiency of microsomal PGE synthase 1 (mPGES1), an enzyme known to couple with COX-2 and convert COX-2-generated PGH_2 into PGE_2, or antagonism of the PGE_2 receptor, EP4, ablates AngII-induced AAA formation in mice.[6, 28, 29] These studies provide further evidence for the role of COX-2-derived PGE_2 in AAA formation.

COX-2 induction in response to AngII has been shown to occur via the activation of several signaling molecules including components of the MAPK pathway such as ERK1/2.[30-32] Furthermore, ERK1/2 activation has also been shown to be important in the formation of AngII-induced AAAs, as inhibition of ERK1/2 activation impairs AAA formation in mice.[33] These findings underscore the importance of COX-2 and ERK1/2 in AAA formation and suggest that activated ERK1/2 (p-ERK1/2) may contribute to COX-2 induction during AAA formation. Because βarr2 is important in AngII-AT1a-mediated ERK1/2 activation, in the present study we investigated whether βarr2 played a role in AngII-induced AAA formation. Using both normolipidemic βarr2-deficient mice and hyperlipidemic ApoE$^{-/-}$/βarr2$^{-/-}$ mice, we demonstrate that βarr2 deficiency significantly attenuates AAA formation. Furthermore, mechanistically it appears that βarr2 mediates AngII-induced COX-2 expression via a p-ERK1/2-dependent pathway and increases the expression of inflammatory markers, thereby contributing to AAA formation.

Methods

Mice

Male, hyperlipidemic ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice at 8-10 weeks of age were use for measuring AngII-induced AAA incidence and severity and for mechanistic experiments. AngII-induced AAA incidence was also examined in 8-month old, male, βarr2$^{+/+}$ and βarr2$^{-/-}$ mice[34] on a normolipidemic C57BL/6 background. Please refer to the supplemental methods section for details.

AAA quantitation

Mice were treated with AngII (1000ng/kg/min) or saline via subcutaneously implanted osmotic mini-pumps and euthanized after 28 days for AAA quantification. A >50% increase in external diameter of the abdominal aorta was used to define the occurrence of an AAA. AAA severity was determined as using a classification scheme described previously[8] and by measuring the wet weights of the abdominal aortas. The presence of an AAA as well as the scoring of AAA pathology was determined by an investigator who was blinded to the mouse genotypes. Upon determination of the AAA incidence and classification, another investigator matched the scored AAAs to the genotypes of the mice.

Blood pressure measurements

Blood pressure was measured in conscious, unrestrained, male ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice by radio-telemetry, before and after treatment with AngII (1000ng/kg/min, osmotic mini-pumps), using methods similar to those described previously.[35, 36] For more details, see the supplemental methods section.

Inhibition of ERK1/2 activation

AngII-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice were treated with the MAPK kinase 1(MEK1) inhibitor, CI1040, as previously described.[33]

Histology and immunohistochemistry

Mice were euthanized at the designated time-points (7 or 28 days) and abdominal aortic sections were stained with hematoxylin and eosin (H&E) or Verhoeff-Van Geisen (VVG, elastin) for histological analysis or with antibodies against COX-2, Mac-3 or p-ERK1/2, for immunohistochemical analysis. Quantitation of immunohistochemistry was performed by determining the ratio of the number of COX-2, p-ERK1/2 or Mac-3 positive cells to the total number of hematoxylin positive nuclei per field (at 400× magnification). For more details, please refer to the supplemental methods section.

Quantitation of mRNA expression

Real-time quantitative PCR analysis for COX-2, CD68, MCP-1, MIP1α, MMP2 and MMP9 was performed using Taqman gene expression assays and the   Ct method with hypoxanthine phosphoribosyl transferase (HPRT) as the endogenous control. For more details, see the supplemental methods section.

Statistics

Data are shown as the mean ± SEM of independent experiments. Fisher's exact test was used to compare AAA incidence among different groups. Two-way ANOVA was use to examine changes in blood pressure between genotypes. One-way ANOVA followed by Bonferroni's multiple comparison tests were used to determine the statistical significance of the data in all other experiments. Values with P 0.05 were considered statistically significant.

Results

βarr2 deficiency attenuates the incidence and severity of AngII-induced AAAs

The effect of βarr2 deficiency on AAA development in hyperlipidemic mice was determined by crossing βarr2$^{-/-}$ mice with hyperlipidemic ApoE$^{-/-}$ mice. AngII infusion for 28 days induced a 61.8% incidence (21 of 34 mice) of AAAs in ApoE$^{-/-}$/βarr2$^{+/+}$ mice, whereas the AAA incidence in ApoE$^{-/-}$/βarr2$^{-/-}$ mice was significantly reduced to 9.5% (2 of 21 mice) (Figure 1A). As expected, AAAs were not observed in the saline-infused ApoE$^{-/-}$/βarr2$^{+/+}$ or ApoE$^{-/-}$/βarr2$^{-/-}$ mice (Figure 1A). The deficiency of βarr2 also attenuated the severity of AngII-induced AAAs, as determined by a classification scheme based on the external diameter of the abdominal aortas (Figure 1B) as well as by measuring the wet weights of the abdominal aortas (Figure 1C). Histological examination of cross-sections of the abdominal aortas from ApoE$^{-/-}$/βarr2$^{+/+}$ mice showed severe thickening and prominent remodeling with thrombi present in the vessel wall (Figure 1D). In contrast, the abdominal aortas from AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ showed minimal thickening and remodeling (Figure 1E), and appeared to be histologically similar to abdominal aortas from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice (Figure 1F). Aortas from saline-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice were histologically indistinguishable from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice (data not shown). These data demonstrate that the deficiency of βarr2 attenuates both AngII-induced AAA incidence and severity in mice on a hyperlipidemic background.

Two previous studies have shown that aged, normolipidemic wild-type C57BL/6 mice (7-11 months) also develop an increased incidence of AAAs when infused with AngII for 28 days; however, the AAA incidence is significantly lower than that observed in hyperlipidemic ApoE$^{-/-}$ mice.[37, 38] Therefore, in addition to studies in the hyperlipidemic ApoE$^{-/-}$ strain, we examined the effect of βarr2 deficiency on AAA incidence in 8-month old, normolipidemic C57BL/6 mice. AngII infusion resulted in a 29.2% incidence (7 of 24 mice) of AAAs in the normolipidemic C57BL/6 βarr2$^{+/+}$ mice (Figure 1G). In contrast, none of the normolipidemic C57BL/6 βarr2$^{-/-}$ mice (0 of 15 mice) displayed any pathology (Figure 1G). Of the 7 C57BL/6 βarr2$^{+/+}$ mice that did develop AAAs, 2 mice displayed a Type 2 aneurysm, 3 mice had a Type 3 aneurysm, and 2 mice displayed aneurysmal rupture. Furthermore, similar to previous studies,[38] the AAAs in the normolipidemic C57BL/6 βarr2$^{+/+}$ mice were grossly similar to those that developed in the hyperlipidemic ApoE$^{-/-}$ mice following AngII infusion. Thus, the deficiency of βarr2 attenuates AngII-induced AAA incidence in both hyperlipidemic and normolipidemic mouse strains. Because the AngII-induced AAA incidence in the C57BL/6 βarr2$^{+/+}$ mice on the normolipidemic background is markedly lower than that observed in the hyperlipidemicApoE$^{-/-}$/βarr2$^{+/+}$ mice (29.2%

versus 61.8%), the mechanistic studies described below were performed in the ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice.

βarr2 deficiency does not alter AngII-induced increases in blood pressure

Previous studies have demonstrated that AngII infusion rapidly increases systolic blood pressure in mice.[39-41] Therefore, to determine whether βarr2-deficiency affected AngII-mediated increases in blood pressure, we measured the systolic blood pressure in ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice. As shown in Table 1, AngII treatment significantly increased the mean systolic blood pressure in both ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice as compared to basal blood pressure. However, there was no significant difference in the systolic blood pressure between ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice at baseline or with AngII treatment (Table 1). These results indicate that the reduction in the AngII-induced AAA incidence in βarr2-deficient mice is not dependent on alterations in blood pressure.

AngII-induced COX-2 expression is attenuated in aortas from βarr2-deficient mice

AngII is known to be a potent inducer of COX-2 expression[30, 31, 42] and our previous studies have indicated that COX-2 contributes to AngII-induced AAA formation.[8, 27] Therefore, to determine whether βarr2 played a role in AngII-induced COX-2 expression, ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice were treated with saline or AngII for 7 or 28 days and analyzed for COX-2 expression. AngII infusion significantly increased the levels of COX-2 mRNA in the aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ mice at experimental days 7 (Figure 2A) and 28 (Figure 2B), as compared to saline infusion. In contrast, there was a significant reduction in AngII-induced COX-2 expression in the aortas of βarr2-deficient mice at both time-points. In addition, numerous COX-2 expressing cells were detected in the medial and adventitial layers of the abdominal aorta from ApoE$^{-/-}$/βarr2$^{+/+}$ mice after 7 days (Figure 2C) and throughout the aneurysmal tissue after 28 days of AngII infusion (Figure 2D). In contrast, significantly fewer COX-2 positive cells were observed in the abdominal aortas from ApoE$^{-/-}$/βarr2$^{-/-}$ mice at both time-points (Figure 2E, 2F and 2G). Thus, βarr2 plays a key role in the AngII-induced COX-2 expression during AAA development.

βarr2 contributes to ERK1/2 activation in AngII-induced AAAs and p-ERK1/2 contributes to COX-2 induction

ERK1/2 signaling is an extensively studied pathway activated by the AT1a-βarr2 complex and p-ERK1/2 is also known to contribute to COX-2 induction in several cell types.[19, 30-32, 43] As shown in Figure 3A, immunohistochemical analysis indicated the presence of p-ERK1/2 in the medial layer of aortas from ApoE$^{-/-}$/βarr2$^{+/+}$ mice after 7 days of AngII infusion. When analyzed at 28 days after AngII infusion, numerous p-ERK1/2 containing cells were observed throughout the aneurysmal aortas from ApoE$^{-/-}$/βarr2$^{+/+}$ mice (Figure 3B). In contrast, in the abdominal aortas from ApoE$^{-/-}$/βarr2$^{-/-}$ mice, significantly reduced p-ERK1/2 was observed at both time-points (Figures 3C, 3D and 3E).

To determine if ERK1/2 activation was involved in the AngII-induced COX-2 expression, we utilized the MEK1 inhibitor, CI1040 that has previously been shown to inhibit ERK1/2 phosphorylation and attenuate AngII-induced AAA formation in mice.[33] In the absence of

CI1040 treatment, AngII infusion for 7 days resulted in a significant induction in COX-2 mRNA in the aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ mice (Figure 3F). However, in mice treated with CI1040 plus AngII, the increase in COX-2 expression was significantly reduced compared to AngII treatment alone. Furthermore, COX-2 mRNA levels following CI1040 plus AngII treatment were not statistically different from those observed in AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice (Figure 3F). Thus, the data indicate that βarr2 is important in AngII-induced ERK1/2 activation, which may subsequently increase COX-2 expression.

βarr2-deficiency attenuates AngII-induced macrophage infiltration into the abdominal aorta

Macrophage infiltration is an important early event during AAA formation.[7] Therefore we examined the role of βarr2 in macrophage infiltration during AngII-induced AAA formation. Immunohistochemical staining for the macrophage marker Mac-3, in the abdominal aortas from AngII-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice indicated an abundant infiltration of macrophages into the media and adventitia by day 7 (Figure 4A), which continued through the 28-day AngII infusion (Figure 4B). However, Mac-3 staining was significantly reduced in the abdominal aortas of ApoE$^{-/-}$/βarr2$^{-/-}$ mice at both time-points, indicating decreased macrophage infiltration (Figures 4C, 4D and 4E). In addition, we observed a robust increase in the mRNA expression for the macrophage marker, CD68, in the aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ mice following AngII infusion (Figure 4F). In contrast, AngII infusion in ApoE$^{-/-}$/βarr2$^{-/-}$ mice did not significantly increase CD68 mRNA expression when compared to saline controls (Figure 4F).

AngII has been shown to stimulate the secretion of inflammatory chemokines such as MCP-1 and MIP1α, which are known to increase macrophage infiltration.[8, 44-47] Therefore, the expression of MCP-1 and MIP1α was examined in the aortas from ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ during the initial stage of AAA formation. AngII infusion for 7 days significantly increased the mRNA expression of both MCP-1 (Figure 4G) and MIP1α (Figure 4H) in the ApoE$^{-/-}$/βarr2$^{+/+}$ aortas as compared to saline infusion. However, the increases in MCP-1 and MIP1α expression in response to AngII were significantly lower in the aortas of ApoE$^{-/-}$/βarr2$^{-/-}$ mice (Figures 4G and 4H). Therefore, the data suggest that attenuated chemokine production may be responsible for reduced macrophage infiltration and diminished AAA formation in ApoE$^{-/-}$/βarr2$^{-/-}$ mice.

βarr2 contributes to increased MMP2 and MMP9 expression during AAA formation

MMPs have been mechanistically implicated in the pathogenesis of AAAs and MMP2 and MMP9 in particular are known to have a concerted role in AAA initiation and progression.[48, 49] As shown in Figures 5A and 5B, AngII treatment significantly increased MMP2 mRNA expression at days 7 and 28 in ApoE$^{-/-}$/βarr2$^{+/+}$ mice. However, in ApoE$^{-/-}$/βarr2$^{-/-}$ mice, the increase in MMP2 expression was significantly lower when analyzed at both the time-points following AngII infusion (Figures 5A and 5B). In contrast to MMP2 expression, AngII treatment resulted in only a modest increase in MMP9 mRNA expression at 7 days in ApoE$^{-/-}$/βarr2$^{+/+}$ mice, and βarr2 deficiency did not appear to significantly alter MMP9 mRNA expression at this time-point (Figure 5C). However, after 28 days of AngII infusion, MMP9 expression was significantly increased in ApoE$^{-/-}$/βarr2$^{+/+}$ mice and βarr2 deficiency attenuated this increase (Figure 5D). Increased MMP expression has been

associated with the proteolytic degradation and elastin breakage that occurs during aneurysmal expansion. Verhoeff-Van Gieson (VVG) staining of abdominal aortic segments from ApoE$^{-/-}$/βarr2$^{+/+}$ mice after 28 days of AngII infusion showed regions of breakage and discontinuity of the medial elastin layer (Figure 5E). In contrast, the elastin fibers in the aortas of AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice appeared intact (Figure 5F), similar to the aortas of saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice (Figure 5G). Aortas from saline-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice were histologically indistinguishable from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice (data not shown).

Discussion

Several studies have demonstrated that AngII infusion leads to the formation of AAAs in mice.[12, 13, 50] AngII exerts its diverse bioactive effects primarily by activating the AT1a receptor in mice, which plays a critical role in AngII-induced AAA development.[17] In addition to mediating traditional G-protein-dependent signaling, the AT1a receptor is also involved in G-protein-independent signaling by forming a complex with the multifunctional scaffolding protein βarr2.[19, 24] In the present study, we investigated a role for βarr2 in AngII-induced AAA formation, and found that βarr2 deficiency significantly attenuates AAA formation in mice in both a hyperlipidemic ApoE background and a normolipidemic C57BL/6 background (Figure 1). These studies suggest that G-protein-independent, βarr2-dependent signaling for the AT1a receptor plays a major role in AngII-induced AAA formation.

Although our current studies showed that βarr2 deficiency attenuated AngII-induced AAA formation in mice on the hyperlipidemic ApoE$^{-/-}$ background, it was not clear whether hyperlipidemia was required for this observed phenotype. Therefore, we examined the effect of βarr2 deficiency on AngII-induced AAA formation in mice on a normolipidemic C57BL/6 background. We have previously reported that 8-10-week old, normolipidemic C57BL/6 mice develop only a 5% incidence of AngII-induced AAAs;[27] however, two recent reports have utilized aged (7-11 month old) C57BL/6 mice and have shown that these mice develop an approximately 40% incidence of AAAs in response to AngII.[37, 38] Therefore, we performed experiments using 8-month old, normolipidemic βarr2$^{+/+}$ and βarr2$^{-/-}$ mice on a C57BL/6 background and found that similar to the effect in ApoE$^{-/-}$ mice, the deficiency of βarr2 also significantly attenuated AngII-induced AAA incidence in the normolipidemic strain (Figure 1G). Thus, the effect of βarr2 deficiency on attenuating AAA incidence is independent of the hyperlipidemic environment. Because we observed only a low AAA incidence (29.2%) in the C57BL/6 βarr2$^{+/+}$ strain, the mechanistic studies in our current report were performed in mice on the ApoE$^{-/-}$ background.

Our previous studies have indicated an important role for COX-2 in AAA formation as genetic deficiency of COX-2 or treatment of mice with the COX-2 selective inhibitor celecoxib, significantly attenuated AngII-induced AAA incidence and severity.[8, 27] Because our present study shows that βarr2 deficiency also attenuates AngII-induced AAA formation, we investigated a possible link between COX-2 and βarr2 in this pathology. Although previous studies have shown that AngII-mediated COX-2 induction can occur via G-protein-dependent AT1a receptor signaling,[30-32] the results in the present study show that

the deficiency of βarr2 attenuates AngII-mediated COX-2 induction. This suggests the involvement of a βarr2-dependent, G-protein-independent mechanism for AngII-mediated COX-2 induction. Our findings are supported by a recent study that showed that treatment of rat vascular SMCs with a 'biased' agonist for the AT1a receptor, which activates G-protein-independent, βarr2-dependent signaling,[20, 51] increased COX-2 expression.[52] Based on these studies, together with the results from our current study, we conclude that AngII-mediated COX-2 induction can indeed occur via a G-protein-independent pathway involving βarr2.

AngII-induced COX-2 expression can occur via activation of components of the MAPK pathway such as ERK1/2,[30-32] and ERK1/2 activation is also known to be involved in AngII-induced AAA formation.[33] Our data show that βarr2 deficiency prevented the AngII-mediated ERK1/2 activation in ApoE^{-/-}/βarr2^{-/-} mice as compared to ApoE^{-/-}/βarr2^{+/+} mice, when examined at either the incipient (7 days) or advanced stage (28 days) of AAA development. Furthermore, similar to the deficiency of βarr2, inhibition of ERK1/2 activation significantly abrogated the AngII-mediated increase in COX-2 expression. Thus, AngII-AT1a-βarr2-mediated COX-2 induction appears to occur, in part, by a mechanism involving activated ERK1/2.

A hallmark of AAA pathology is macrophage infiltration into the aorta, which is an early cellular event following AngII infusion that continues throughout the development of the disease.[7, 53] Our previous studies using the COX-2-deficient mice and more recent studies using an EP4-selective antagonist have suggested that COX-2-derived PGE_2 stimulates the production of inflammatory chemokines such as MCP-1 and MIP1α, which contribute to macrophage infiltration during AAA development.[6, 8, 29] Because βarr2 has been shown to contribute to pro-inflammatory gene expression in other models of inflammation,[22, 54] we examined the role of βarr2 in the inflammatory response that occurs during AAA formation. The observations that βarr2 deficiency attenuated the expression of COX-2 and the inflammatory chemokines, MCP-1 and MIP1α, together with reducing macrophage infiltration, suggest that βarr2 positively regulates inflammation in AngII-induced AAAs through the induction of COX-2 expression.

Proteolytic degradation within the vessel wall mediated largely by MMPs is a common feature of AAAs, wherein elastin fragmentation in the medial and adventitial layers is believed to contribute to the initial dilation, and increased collagenase activity may be responsible for aneurysmal rupture.[55] A preponderance of evidence in experimental models as well as in humans supports a role for MMPs, particularly MMP2 and MMP9, in AAA initiation and progression.[10, 11, 48, 56] Our studies show that the deficiency of βarr2 attenuated the increase in MMP2 when analyzed at 7 and 28 days following AngII infusion. However, the effect of βarr2 deficiency on MMP9 expression was prominent only at the late stages of aneurysm development (Figure 5D). These results may be explained by previous studies suggesting an important role for MMP2 during the formative stage of AAAs involving macrophage infiltration, and a dominant role for MMP9 during the progressive stage of AAAs.[5]

AngII is known to be a potent inducer of hypertension in mice.[39-41] However, it has also been shown that administration of hydralazine to reduce AngII-induced hypertension does

not affect the formation of AAAs, indicating that AngII-induced AAA formation is independent AngII-induced hypertension.[57] Our studies show that βarr2 deficiency had no effect on the hypertensive response to AngII (Table 1). Thus, these results suggest that our observed effect of βarr2 deficiency on reducing AAA formation is independent of alterations in blood pressure.

In summary, these studies demonstrate that βarr2 plays a crucial role in the development of AngII-induced AAAs in mice via the induction of COX-2 expression. Based on our current data and previous studies, we propose the following mechanism for βarr2-dependent AAA formation. Activation of AT1a by AngII leads to βarr2 recruitment and AT1a-βarr2 complex formation, which results in the activation of ERK1/2 and COX-2 induction. The AT1a-βarr2-dependent increase in COX-2 expression contributes to AAA initiation via the induction of inflammatory chemokines and resultant recruitment of macrophages, and this combined with increased MMP expression exacerbates AAA pathology. Thus, our studies identify βarr2 as a novel target for the design of pharmacotherapies for AAAs. The fact that βarr2-dependent and G-protein-dependent signaling pathways are pharmacologically distinguishable has given rise to the concept of 'biased agonism', whereby a ligand may selectively trigger one or the other pathway.[24, 58] Based on this emerging concept, future studies using 'biased' ligands to target βarr2-mediated signaling may provide further mechanistic insight into the contribution of βarr2 to AAA formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations

AAA	abdominal aortic aneurysm
AngII	angiotensin II
ApoE	apolipoprotein E
AT1a	angiotensin II type 1a
βarr1	β-arrestin-1
βarr2	β-arrestin-2
COX-2	cyclooxygenase-2
DAB	di-amino benzidine
ERK1/2	extracellular signal-regulated kinase 1/2
GPCR	G protein coupled receptor
H&E	hematoxylin and eosin
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MEK1	MAPK kinase 1
MIP1α	macrophage inflammatory protein 1α
MMP	matrix metalloproteinase
mPGES1	microsomal prostaglandin E synthase 1
p-ERK1/2	phosphorylated ERK1/2
PGE$_2$	prostaglandin E$_2$
VVG	Verhoeff-Van Geisen

Novelty and Significance

What Is Known?

- Angiotensin II (AngII) infusion results in the formation of abdominal aortic aneurysms (AAAs) in mice by activating the AT1a receptor and inducing an inflammatory response.

- Inflammatory mediators produced by cyclooxygenase-2 (COX-2) are key contributors to AngII-induced AAA pathology.

- β-arrestin 2 (βarr2) activates extracellular signal-regulated kinase 1/2 (ERK1/2) signaling by coupling with AT1a, and ERK1/2 activation has been shown independently to be important in AAA development.

What New Information Does This Article Contribute?

- βarr2 is critical for the development of AngII-induced AAAs in mice.

- βarr2 mediates AngII-induced AAA formation by inducing COX-2 expression via the ERK1/2 pathway and by increasing inflammation.

AAAs are a life-threatening vascular condition for which no pharmacological treatments are currently available. Hence, a basic understanding of the mechanisms that contribute to AAA formation is required to develop better treatments for this condition. A commonly used mouse model to study AAA formation involves infusion of AngII, which leads to adverse cardiovascular effects primarily by activating the G-protein coupled receptor (GPCR), AT1a. Recent studies have shown that the multifunctional scaffolding protein βarr2 forms a complex with GPCRs such as AT1a to initiate G-protein-independent signaling and contribute to many pathologies. Herein, we show that βarr2 deficiency attenuates AngII-induced AAAs in mice, suggesting a role for G-protein-independent signaling by the AT1a receptor in AAA formation. Our work provides evidence that βarr2 contributes to AngII-induced AAA development by activating ERK1/2 signaling andinducing the pro-inflammatory enzyme - COX-2. These results suggest that βarr2 may be a novel target in designing pharmacological treatments for AAAs.
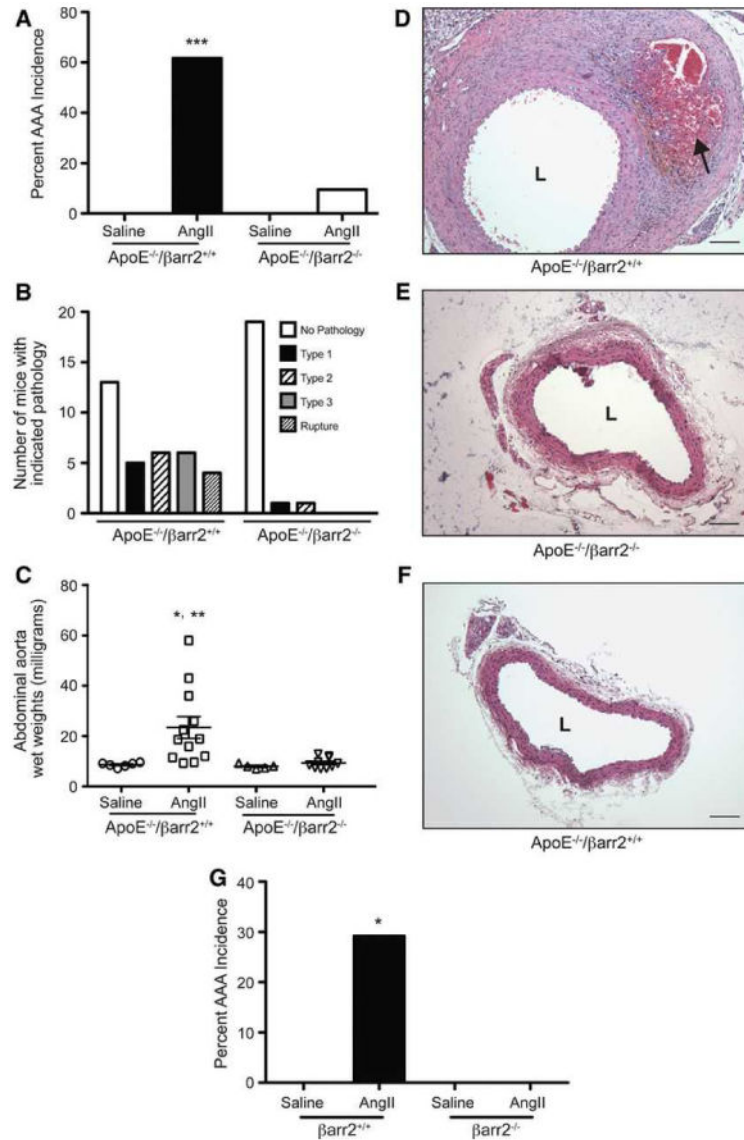
Figure 1. AngII-induced AAA formation in hyperlipidemic ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice and normolipidemic C57BL/6 βarr2$^{+/+}$ and βarr2$^{-/-}$ mice

A) Percentage of AAA incidence following 28 days of AngII infusion in ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice. ***, significantly different from saline treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice and AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, $P<0.001$, N   10 per group. **B)** AAAs in ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice were scored from Type 1 to Type 4 pathology, based on the external diameter using a classification scheme similar to the one described previously[18] **C)** AAA severity in in ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice as determined by abdominal aorta wet weights. Each symbol represents an individual animal. *, significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice, $P<0.05$. **, significantly different from AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, $P<0.01$, N   5 per group. Representative images of H&E-stained aortic sections from **D)** AngII-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice **E)** AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ and **F)** saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice. Arrow indicates thrombus formation. Scale bars, 0.1mm. L, lumen. **G)** Percentage of AAA incidence in

normolipidemic C57BL/6 βarr2$^{+/+}$ and $^{-}$βarr2$^{-/-}$ mice following 28 days of AngII infusion. *, significantly different from saline-treated βarr2$^{+/+}$ mice and AngII-treated βarr2$^{-/-}$ mice, $P<0.05$, N 10 per group.
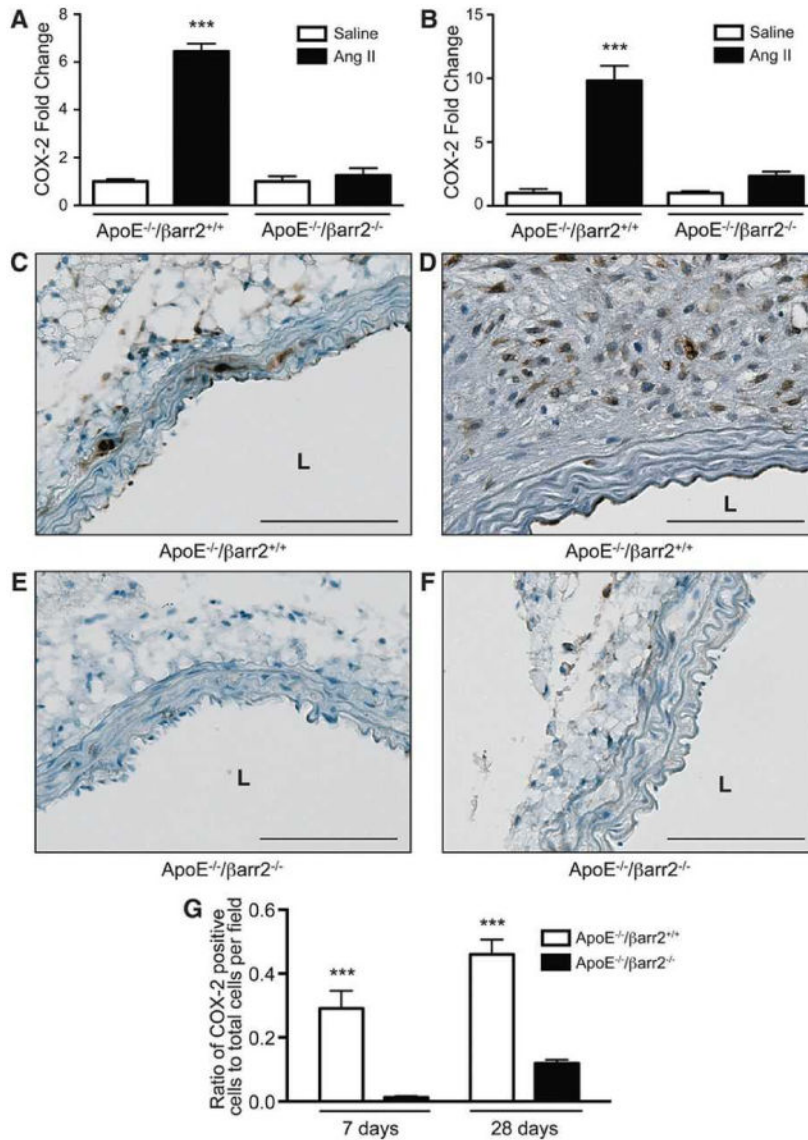
Figure 2. COX-2 expression is attenuated in the aortas of βarr2-deficient mice

Quantitative PCR analysis of COX-2 mRNA expression in the aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice at **A)** 7 days and **B)** 28 days following AngII or saline infusion. ***, significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ and AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice. Data represent mean ± SEM, $P<0.001$, N 6 per group. Representative photomicrographs of COX-2 immunostaining in abdominal aortic segments from ApoE$^{-/-}$/βarr2$^{+/+}$ mice at **C)** 7 and **D)** 28 days after AngII infusion and ApoE$^{-/-}$/βarr2$^{-/-}$ mice at **E)** 7 and **F)** 28 days following AngII infusion. Brown staining (DAB) indicates COX-2 expression and sections are counterstained with hematoxylin (blue). Scale bars, 0.1mm. L, lumen. **G)** Quantitation of COX-2 immuno-positive cells. ***, significantly different from ApoE$^{-/-}$/βarr2$^{-/-}$ mice. Data represent mean ± SEM, $P<0.001$, N = 5 per group.
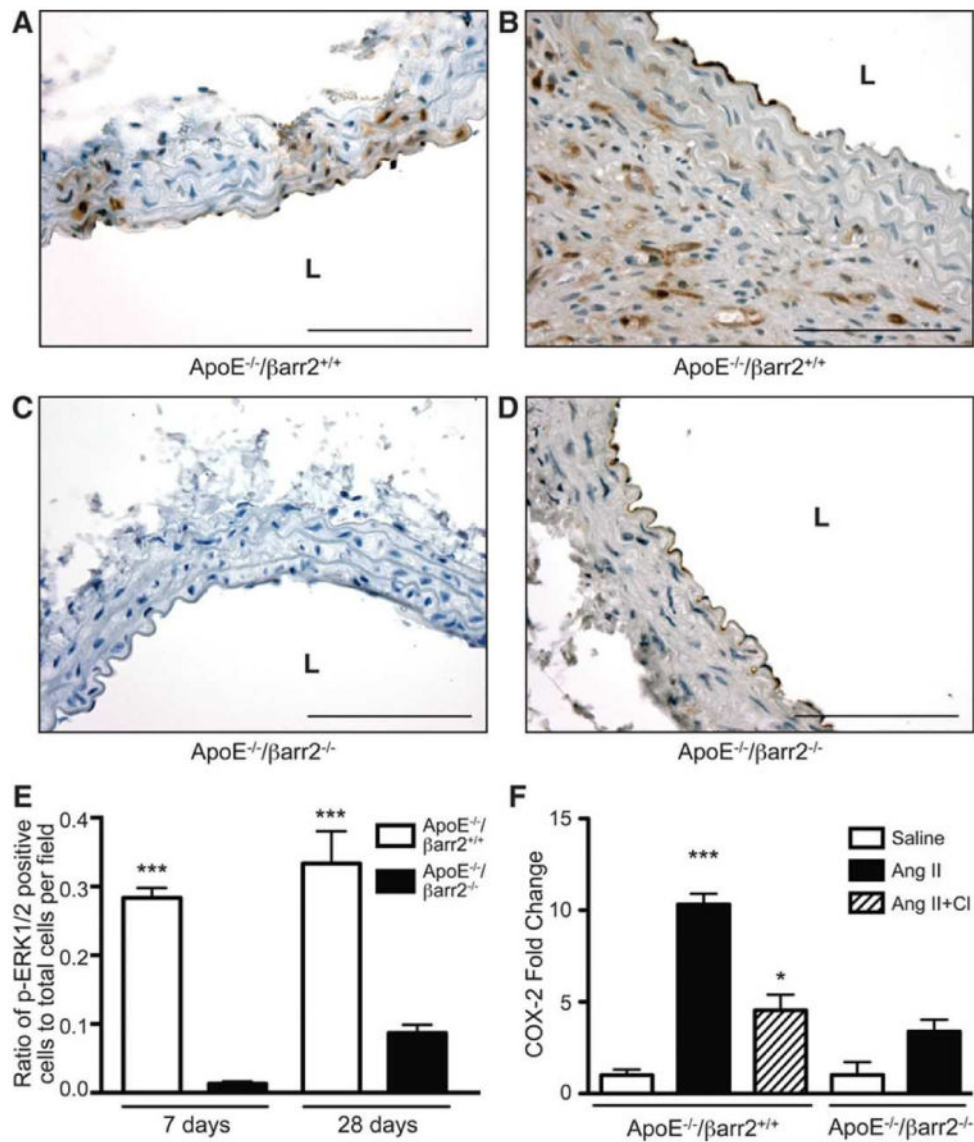
Figure 3. βarr2 deficiency results in attenuated ERK1/2 activation

Representative photomicrographs of p-ERK1/2 immunostaining in abdominal aortic segments from ApoE$^{-/-}$/βarr2$^{+/+}$ mice at **A)** 7 and **B)** 28 days after AngII infusion and ApoE$^{-/-}$/βarr2$^{-/-}$ mice at **C)** 7 and **D)** 28 days following AngII infusion. Brown staining indicates p-ERK1/2 and sections are counterstained with hematoxylin (blue). Scale bars, 0.1mm. L, lumen **E)** Quantitation of p-ERK1/2 immuno-positive cells. ***, significantly different from ApoE$^{-/-}$/βarr2$^{-/-}$ mice. Data represent mean ± SEM, $P<0.001$, N = 5 per group **F)** Quantitative PCR analysis of COX-2 mRNA expression in abdominal aortas from ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice following treatment with saline, AngII or AngII plus CI1040 (CI, MEK1 inhibitor) for 7 days. ***, significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice, $P<0.001$, *, significantly different from AngII-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice, $P<0.05$, Data represent mean ± SEM, N ≥ 5 per group. COX-2 mRNA levels in AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice were not significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice or AngII plus CI treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice.
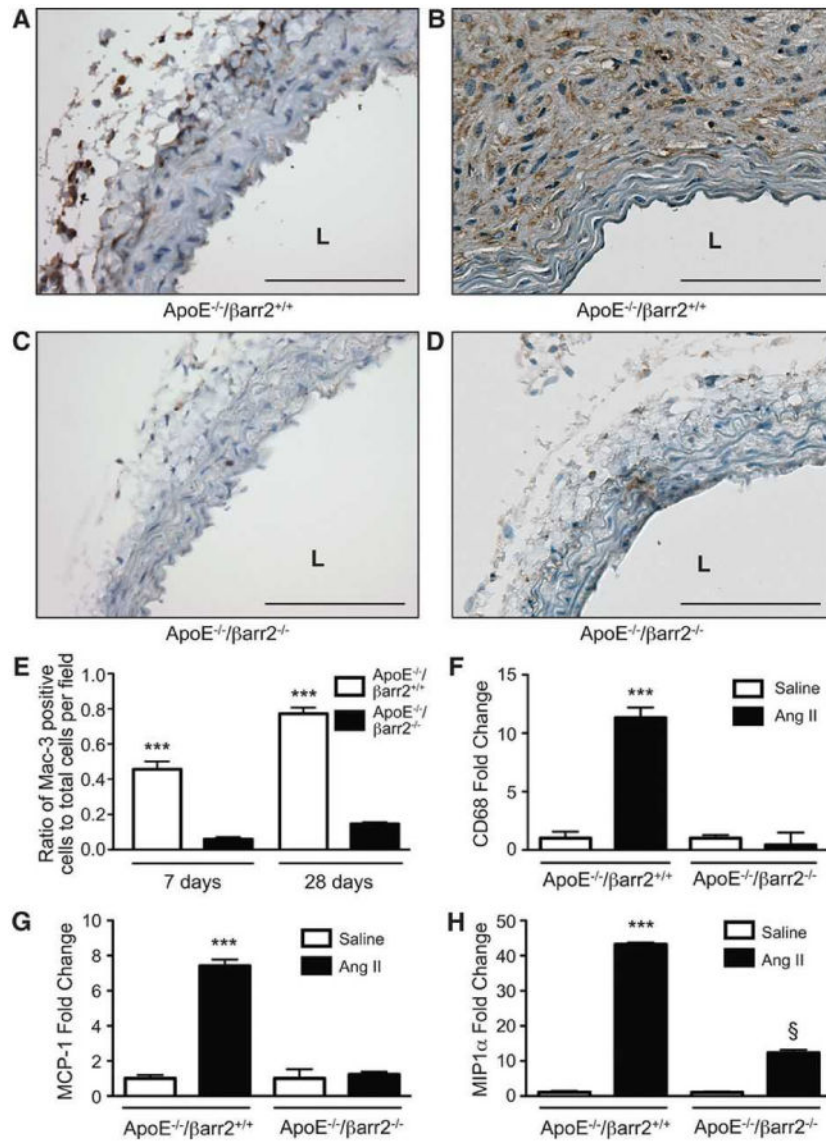
Figure 4. βarr2 contributes to macrophage infiltration into the aorta following AngII infusion
Representative photomicrographs of Mac-3 immunostaining in abdominal aortic segments
from ApoE$^{-/-}$/βarr2$^{+/+}$ mice at **A)** 7 and **B)** 28 days after AngII infusion and ApoE$^{-/-}$/βarr2$^{-/-}$
mice at **C)** 7 and **D)** 28 days after AngII infusion. Brown staining shows Mac-3 expression
and sections are counter-stained with hematoxylin (blue). Scale bars, 0.1mm. L, lumen. **E)**
Quantitation of Mac-3 immuno-positive cells. ***, significantly different from ApoE$^{-/-}$/
βarr2$^{-/-}$ mice. Data represent mean ± SEM, $P<0.001$, N = 5 per group. **F)** Quantitative PCR
analysis CD68 mRNA expression in the abdominal aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/
βarr2$^{-/-}$ mice following treatment with saline or AngII for 7 days. ***, significantly different
from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ and AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, $P<0.001$. Data
represent mean ± SEM, N   6 per group. **G)** Quantitative PCR analysis of MCP-1 mRNA
expression in the abdominal aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice following
treatment with saline or AngII for 7 days. ***, significantly different from saline-treated
ApoE$^{-/-}$/βarr2$^{+/+}$ and AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, $P<0.001$. Data represent mean ±

SEM, N 6 per group. **H)** Quantitative PCR analysis of MIP1α mRNA expression in the abdominal aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice following treatment with saline or AngII for 7 days. ***, significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ and AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, *P*<0.001. §, significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, *P*<0.001. Data represent mean ± SEM, N 6 per group.
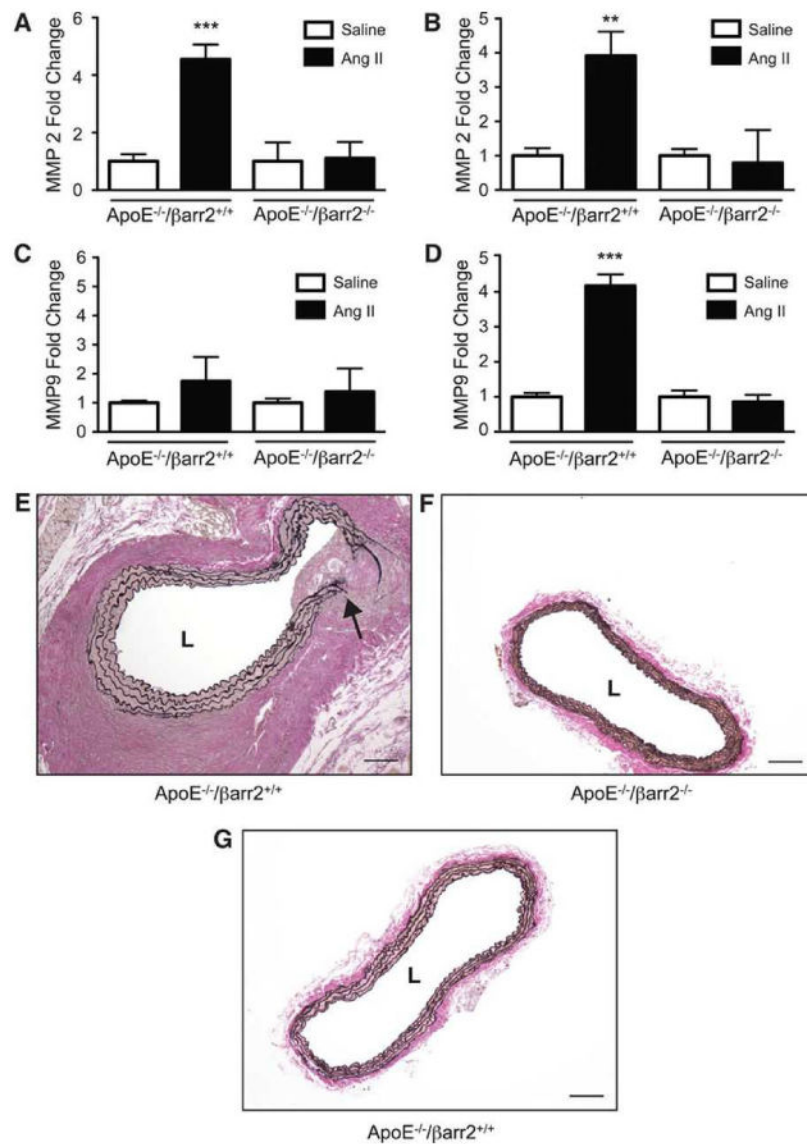
Figure 5. MMP expression is attenuated in the aortas of βarr2-deficient mice

Quantitative PCR analysis of MMP2 mRNA expression in the abdominal aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice at **A)** 7 days or **B)** 28 days following AngII or saline infusion. ***, significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ and AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, $P<0.001$, **, significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ and AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, $P<0.01$. Data represent mean ± SEM, N 6. Quantitative PCR analysis of MMP9 mRNA expression in the abdominal aortas of ApoE$^{-/-}$/βarr2$^{+/+}$ and ApoE$^{-/-}$/βarr2$^{-/-}$ mice at **C)** 7 days or **D)** 28 days following AngII or saline infusion. ***, significantly different from saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ and AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ mice, $P<0.001$. Data represent mean ± SEM, N 6. Representative images of VVG stained abdominal aortic sections from **E)** AngII-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice. Arrow indicates breakage in the elastin layer **F)** AngII-treated ApoE$^{-/-}$/βarr2$^{-/-}$ and **G)** saline-treated ApoE$^{-/-}$/βarr2$^{+/+}$ mice. Scale bars, 0.1mm. L, lumen.

Table 1

Systolic Blood Pressure

	ApoE$^{-/-}$βarr2$^{+/+}$	ApoE$^{-/-}$/βarr2$^{-/-}$
Baseline (mmHg)	123.5 ± 7.1	122.9 ± 3.9
AngII (mmHg)	153.3 ± 7.2 *	154.9 ± 10.2 *

Data represent the mean ± SEM,

*, significantly different from baseline, P < 0.0001, N 4 per group.