Andrographolide Ameliorates Abdominal Aortic Aneurysm Progression by Inhibiting Inflammatory Cell Infiltration through Downregulation of Cytokine and Integrin Expression

Jun Ren, Zhenjie Liu, Qiwei Wang, Jasmine Giles, Jason Greenberg, Nader Sheibani, K. Craig Kent, and Bo Liu

Division of Vascular Surgery, Department of Surgery (J.R., Z.L., Q.W., J.Gi., J.Gr., K.C.K., B.L.) and Department of Ophthalmology and Visual Sciences (N.S.), University of Wisconsin, Madison, Wisconsin; And Department of Vascular Surgery, 2nd Affiliated Hospital School of Medicine, Zhejiang University, Zhejiang, China (Z.L.)

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ABSTRACT

Abdominal aortic aneurysm (AAA), characterized by exuberant inflammation and tissue deterioration, is a common aortic disease associated with a high mortality rate. There is currently no established pharmacological therapy to treat this progressive disease. Andrographolide (Andro), a major bioactive component of the herbaceous plant Andrographis paniculata, has been found to exhibit potent anti-inflammatory properties by inhibiting nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) activity in several disease models. In this study, we investigated the ability of Andro to suppress inflammation associated with aneurysms, and whether it may be used to block the progression of AAA. Whereas diseased aortae continued to expand in the solvent-treated group, daily administration of Andro to mice with small aneurysms significantly attenuated aneurysm growth, as measured by the diminished expansion of aortic diameter (165.68 ± 15.85% vs. 90.62 ± 22.91%, *P < 0.05). Immunohistochemistry analyses revealed that Andro decreased infiltration of monocytes/macrophages and T cells. Mechanistically, Andro inhibited arterial NF-κB activity and reduced the production of proinflammatory cytokines [CCL2, CXCL10, tumor necrosis factor α, and interferon-γ] in the treated aortae. Furthermore, Andro suppressed α4 integrin expression and attenuated the ability of monocytes/macrophages to adhere to activated endothelial cells. These results indicate that Andro suppresses progression of AAA, likely through inhibition of inflammatory cell infiltration via downregulation of NF-κB-mediated cytokine production and α4 integrin expression. Thus, Andro may offer a pharmacological therapy to slow disease progression in patients with small aneurysms.

Introduction

Abdominal aortic aneurysm (AAA), the progressive weakening and dilatation of the abdominal aorta, is a major aortic disease associated with a high mortality rate. It occurs most commonly in those over 50 years old, in men, and among those with a family history of the disease (Kent, 2014). As the elderly population increases, AAA becomes a more serious health problem, especially in Western nations. In the United States, the total number of AAAs remains stable at 45,000 cases per year (Dua et al., 2014). The majority of these new cases are small AAAs (defined as less than 5.5- and 5-cm aortic diameter in men and women, respectively) and largely asymptomatic. If left untreated, however, the continuing extension and thinning of the vessel wall may eventually result in a sudden unpredictable rupture. Ruptured AAAs are responsible for at least 13,000 deaths in the United States and 152,000 deaths worldwide annually (Kent, 2014; GBD 2013 Mortality and Causes of Death Collaborators, 2015). Unfortunately, an effective pharmacological treatment of AAAs is not currently available. Moreover, surgical treatment of small aneurysms is not usually recommended due to a low benefit-to-risk ratio. As a result, patients with small aneurysms are left untreated and in a “watchful waiting” program to monitor AAA progression (Filardo et al., 2012; Bown et al., 2013).

The clinical need for effective and safe drugs to slow aneurysm progression in patients with small AAAs has motivated active studies on aneurysm pathophysiology. Intense inflammation, implied by excessive cytokines and massive infiltrated inflammatory cells, is a major pathologic feature of AAAs, which has been replicated in various animal models of AAA (Daugherty and Cassis, 2004; Shimizu et al.,...
To fully recover from anesthesia. Seven days after aneurysm induction, mice were randomly assigned to two groups: (1) solvent (DMSO) control and (2) Andro. Immediately prior to i.p. injection, 3 μl of Andro (dissolved in DMSO at 50 mg/ml) was diluted with 200 μl of saline. An equal volume of DMSO was diluted and injected accordingly. We chose postoperative day 7 to begin treatment because aneurysm dilations at this time are small but significant (Supplemental Fig. 1, A and B) (Miyake et al., 2007; Liu et al., 2014). Daily i.p. injection of DMSO to mice produced an insignificant effect on aneurysmal dilation compared with mice not receiving DMSO (165.68 ± 15.85% vs. 166.67 ± 15.46%, P < 0.05 in elastase-treated arteries postoperative at day 14; and 43.54 ± 7.24% vs. 43.62 ± 5.36%, P > 0.05 in inactive elastase-treated arteries postoperative at day 14). The in vivo dosage of Andro in the literature ranges from 1 to 100 mg/kg body weight, with 5 mg/kg body weight (used in the current study) as a commonly used dosage, which is considerably lower than the LD₅₀ for intraperitoneally administered andrographolide (11.6 g/kg body weight) (Handa and Sharma, 1990; Wang et al., 2007; Hsieh et al., 2011; Zhu et al., 2013). At the selected time points, mice were euthanized by CO₂ inhalation. The external diameter of the largest portion of abdominal aorta was measured and used to calculate the percentage increase in maximal external aortic diameter compared to the diameter recorded prior to the elastase perfusion. Tissues intended for RNA isolation were harvested and stored in RNAlater RNA Stabilization Reagent (Qiagen, Valencia, CA). Tissues intended for immunohistochemistry were imbedded in OCT Compound (Sakura, Alphen aan den Rijn, Netherlands). Frozen sections were cut to 6 μm thick using a Leica CM3050S cryostat (Buffalo Grove, IL). All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison (protocol M02284) and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (National Research Council, 2011).

**Cell Culture and In Vitro Treatment.** Primary mouse aortic SMCs were isolated from arteries of C57BL/6J mice as previously described (Clowes et al., 1989; Lengfeld et al., 2012). Primary SMCs were grown at 37°C in 5% CO₂ in DMEM modified to contain 4 mM L-glutamine, 1 g/l D-glucose, and 110 mg/l sodium pyruvate (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. Cells between passages three and seven were used. The mouse monocyte/macrophage cell line RAW264.7 (RAW) was obtained from American Type Culture Collection (Manassas, VA) and was maintained as recommended in DMEM modified containing 4.5 g/l D-glucose (Life Technologies) supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. Mouse aortic endothelial cells (ECs) were isolated from male C57BL/6J mice, as described previously (Su et al., 2003). ECs were grown on 1% gelatin-coated dishes in DMEM containing 20% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml (Sigma-Aldrich), 100 μg/ml endothelial growth supplement (Sigma-Aldrich), and murine recombinant interferon-γ (R&D Systems) at 44 U/ml (Su et al., 2003). For in vitro treatment, a stock solution of 15 μM was prepared by dissolving Andro in DMSO, then diluted to desired concentrations with medium immediately prior to experiments. The final concentration of DMSO was 0.1%, which showed no effects by itself (Fig. 5, B–E; Supplemental Figs. 2, A–G and 3, A–G). Andro, at the final concentration of 15 μM, has been reported to effectively inhibit NF-κB in ECs and SMCs (Xia et al., 2004; Ren et al., 2014), whereas the dosage of Andro for treating macrophages is less established. To determine the in vitro dosage, we conducted dose-response studies using RAW264.7 cells did not significantly decrease cell viability below a dose of 30 μM (Supplemental Fig. 4A). Preincubation of RAWs with 15 or 20 μM Andro reduced adhesion to the fibronectin-coated surface by 70 and 80%, respectively. However, differences in adhesion inhibition caused by 15 and 20 μM did not reach significance (Supplemental Fig. 4B), suggesting 15 μM Andro
caused close-to-maximal inhibition on adhesion of monocyte/macrophages without affecting viability. Therefore, we conducted the rest of the in vitro studies with 15 μM Andro.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction. Total RNA was extracted from cultured cells or mouse aortic arteries using Trizol reagent (Life Technologies) according to the manufacturer’s protocols. Two micrograms of RNA was used for the first-strand cDNA synthesis (Applied Biosystems, Carlsbad, CA). A no-reverse-transcriptase control was included in the same polymerase chain reaction (PCR) mixtures without reverse transcriptase to confirm the absence of DNA contamination in RNA samples. The quantitative real-time PCR primers for Ccl2, Ccl5, Ccl7, Ccl10, Ccl16, Tnf, Ifng, and Gsdp were purchased from Qiagen. Primers for amplification of integrins were Igα4 (forward: 5’-GAATCCAAAAACAGCCTCGCA-3’; reverse: 5’-TGACGTTAGCAATGCCAGTG-3’), Igα3L (forward: 5’-ATGCAACAGTACAGGCACG-3’; reverse: 5’-TGGGCTGAGCTGATTTGCTAC-3’), and Igβ2 (forward: 5’-CAGGATCCACAAATGGCCTACAG-3’; reverse: 5’-CTCGGCTGAGTGAATGCC-3’) (Ip et al., 2007). Reactions were carried out in 96-well optical reaction plates using SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primers in a 7500 Fast Real-Time PCR System (Applied Biosystems). Amplification of each sample was analyzed by melting curve analysis, and relative differences in each PCR sample were corrected using glyceraldehyde-3-phosphate dehydrogenase mRNA as an endogenous control, and the ΔΔct method. ΔCt = ΔCt(target gene) - ΔCt(loading control).

CellTiter-Glo Luminescent Cell Viability Assay. RAWs were seeded in 96-well plates at a density of 3 × 104 cells per well. Twenty-four hours later, the medium was replaced with fresh growth medium containing Andro or DMSO. After culturing for 48 hours, CellTiter-Glo Reagent was added. Luminescence was recorded 10 minutes after reagent addition using a FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA).

Cell Adhesion Assay. Adhesion to a fibronectin-coated surface was evaluated in 96-well plates as described previously (Liu et al., 2015). In brief, RAWs pretreated with Andro or DMSO were seeded in fibronectin-coated wells and incubated at 37°C for 45 minutes. After three washes with phosphate-buffered saline (PBS), cells were fixed in 4% paraformaldehyde, then stained with crystal violet (5 mg/ml in 2% BSA) for 1 hour. After washing PBS, stained tissue sections were visualized with a Nikon Eclipse Ti inverted microscope system, and digital images were acquired using a Nikon DS-Ri1 digital camera. Quantification of stains was performed with ImageJ software as previously described.

Results

Andrographolide Inhibits Progression of Abdominal Aortic Aneurysm. We tested Andro in a murine model of AAA, which used a brief (5 minutes) intraluminal perfusion of the abdominal aorta with porcine pancreas elastase. The control mice were subjected to identical surgical manipulation, but were perfused with heat-inactivated elastase. With time, the elastase-treated group exhibited continuous aortic expansion (Supplemental Fig. 1, A and B), which was accompanied by the appearance of typical aneurysmal pathologic features, including inflammation, fibrosis, and elastin degradation (Liu et al., 2014, 2015; Wang et al., 2015). In contrast, aorta treated with inactivated elastase showed neither continuous aortic expansion nor tissue deterioration (Supplemental Fig. 1, A and B).

To evaluate the therapeutic potential of Andro, we selected day 7 post elastase perfusion as the starting point for drug treatment. At this time point, the aortic expansion was small but significantly larger than arteries perfused with inactivated elastase (Supplemental Fig. 1, A and B). Furthermore, inflammation as measured by monocyte and macrophage accumulation in the aortic tissue had clearly developed (Liu et al., 2015). The elastase-treated mice were randomly divided into two groups, one treated daily with solvent (DMSO) and the other with Andro (5 mg/kg body weight) (Fig. 1A). A separate group of mice that were perfused with inactivated elastase and then treated with solvent were used as a baseline control. Mice were sacrificed 14 days post aneurysm induction. Elastase-perfused solvent-treated aortae predominantly appeared dilated and inflamed, whereas elastase-perfused Andro-treated aortae appeared normal, which resembled arteries perfused with inactivated elastase (Fig. 1B). We quantified the inhibitory effects of Andro on aortic dilation by calculating the percentage increase in maximum aortic diameter. As shown in Fig. 1B, Andro markedly decreased the aortic expansion compared with the solvent-treated mice (90.62 ± 22.91% vs. 165.68 ± 15.85%, P < 0.05). Histologically, administration of Andro preserved vascular tissue...
Andrographolide Inhibits NF-κB Activation during AAA Pathogenesis. Since Andro has been implicated to act on the NF-κB pathway, we investigated whether Andro affects NF-κB activation in aneurysm using an antibody that recognizes the DNA-binding or activated form of p65 (Aoki et al., 2007). Prior to Andro treatment, activated p65 was readily detectable in the aortic wall 7 days after elastase perfusion, mostly in cells that were positive for smooth muscle α actin (an SMC marker) as well as in cells positive for MOMA-2 (a monocyte and macrophage marker) (Fig. 2, A and B). Activation of NF-κB persisted in the aortic wall for at least 14 days post aneurysm induction (Fig. 2C). The 1-week Andro treatment (days 7–13) nearly eliminated NF-κB activation (Fig. 2C).

Andrographolide Attenuates AAA-Associated Inflammatory Cytokine Expression. To further investigate how Andro halts aneurysm progression, we turned to cultured SMCs and macrophages, two primary sources of inflammatory cytokine during AAA pathogenesis. To mimic inflammation associated with AAA, we treated SMCs isolated from the mouse aorta with TNFα (10 ng/ml), which caused a rapid and sustained phosphorylation of p65 at Ser536 as well as degradation of IκB (Fig. 3, A and B). Pretreating SMCs with Andro (15 μM) greatly attenuated p65 phosphorylation but did not significantly alter IκB degradation (Fig. 3, A and B). Next, we examined whether Andro inhibits the expression of NF-κB target genes (in this case, chemokines and cytokines found in aneurysmal tissues). As shown in Fig. 3, C–H, TNFα stimulated expression of Ccl2, Ccl5, Ccl7, Cxcl10, Cxcl16, and Tnf in SMCs, ranging from 10- to 160-fold. Compared with DMSO, Andro significantly decreased production of these inflammatory cytokines (Fig. 3, C–H). Interestingly, expression of Ifng in SMCs was not affected by TNFα or Andro treatment (Fig. 3I).

Although Andro effectively blocked basal levels of cytokine and chemokine expression in RAWs, this monocyte/macrophage cell line was not as responsive to TNFα (10 ng/ml) as SMCs (Supplemental Fig. 5, A–G). Since toll-like receptor signaling plays a crucial role in pathogenesis of aneurysms (Vorkapic et al., 2015), we used the well characterized toll-like receptor ligand LPS (100 ng/ml) as a stimulus. Andro decreased LPS-induced p65 phosphorylation at Ser536 without affecting IκB in RAWs (Fig. 4, A and B). Moreover, Andro reduced the LPS-induced expression of Ccl2, Ccl5, Ccl7, Cxcl10, and Tnf by 40–80% (Fig. 4, C–F and H). Of note, expression of Cxcl16 and Ifng in RAWs was not sensitive to LPS, even though Andro decreased basal levels of these inflammatory cytokines (Fig. 4, G and I).

Andrographolide Decreases Monocyte/Macrophage Adhesion. Firm adhesion of monocytes/macrophages to the endothelium is another crucial event leading to vascular inflammation. To simulate this early inflammatory step, we used an in vitro adhesion assay in which fluorescence-labeled RAWs were allowed to adhere to a monolayer of ECs for 20 minutes. After washing vigorously, adhered RAWs were imaged and counted. Activation of ECs with TNFα (10 ng/ml) increased the number of adhered RAWs by 130% (Fig. 5A). Pretreating ECs, RAWs, or both cell types with Andro resulted in significantly reduced adhesion of RAWs. However, treating RAWs or both cell types with Andro produced more profound inhibition than treating EC alone (Fig. 5A).

NF-κB is known to regulate endothelial expression of adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Wang et al., 2007). In comparison, the underlying mechanism of Andro on macrophage adhesion is less understood. Very late antigen 4 (also known as αβδ integrin) and lymphocyte function–associated antigen 1 (also known as αLβ2 integrin) are major integrins on the monocyte/macrophage surface that interact with VCAM-1 and ICAM-1 during adhesion. Therefore, we tested whether Andro affects integrin expression in RAWs. As shown in Fig. 5B, treatment of RAWs with Andro for 6 or 12 hours reduced α4 (Itgal4) integrin expression by 57 and 58%, the expression of β1 (Itgb1), αL (Itgal), and β2 (Itgb2) integrins were not significantly affected (Fig. 5, C–E).

Andrographolide Inhibits Inflammatory Cytokine Production and Inflammatory Cell Infiltration in Aneurysm. Having established the inhibitory role of Andro in cultured SMCs and macrophages, we re-examined mouse
aneurysm tissues. Consistent with the literature, expression of AAA-associated inflammatory cytokines, including Tnf, Ifng, Ccl2, Ccl5, Ccl10, and Cxcl16, was greatly increased in the elastase-perfused arterial wall as compared with heat-inactivated elastase-treated arteries (Fig. 6, A–G). Daily administration of Andro 7 days after aneurysm induction significantly inhibited expression of Ccl2, Cxcl10, Tnf, and Ifng as compared with solvent treatment (Fig. 6, A–D). Interestingly, Ccl5, Ccl7, and Cxcl16 mRNA expression was not significantly affected by Andro treatment (Fig. 6, E–G).

We next examined vascular infiltration of inflammatory cells. Immunohistochemistry with antibodies against MOMA-2 (a monocyte and macrophage maker), CD3 (a T cell marker), and Ly6G (a neutrophil marker) revealed that infiltration of monocytes/macrophages as well as T cells were significantly decreased by Andro treatment (Fig. 7, A and B). The number of Ly6G+ cells were comparable between Andro and solvent treatment groups. Of note, at day 14 after aneurysm induction, Ly6G+ cells constituted less than 1% of arterial wall cells (Fig. 7, A and B).

**Discussion**

Chronic inflammation has long been recognized as a crucial pathologic event in AAAs. However, its underlying molecular and cellular mechanisms remain incompletely understood. Experimental evidence published by several investigative groups, including our own, suggests active interplays between infiltrating macrophages and resident SMCs (Curci and Thompson, 2004; Liu et al., 2015). Vascular SMCs are a major source of extracellular matrix proteins that render elastic property and tensile strength to the aortic wall. On the other hand, injured SMCs actively recruit inflammatory cells by producing chemokines. If SMCs are considered as the soil of inflammation, the recruited inflammatory cells, particularly M1 macrophages, are inflammatory amplifiers and tissue
destroyers. They amplify inflammation by releasing cytokines (Curci and Thompson, 2004), destroy tissues by producing matrix-degrading enzymes such as matrix metalloproteinases (Thompson et al., 1995; Longo et al., 2002), and eliminate SMCs through proapoptotic or necrotic signals, including reactive oxygen species, TNFα, and Fas ligand (Li et al., 1997; Wang et al., 2014). As such, anti-inflammatory strategies, including those that deplete neutrophils or neutralize cytokines, such as TNFα, preserve smooth muscle and reduce matrix proteolysis (Eliason et al., 2005; Jayaraman et al., 2008). Evidence presented here proved that inhibition of inflammation through systematic administration of Andro blocked aneurysm progression in mice, evidenced by halted aortic expansion, reduced elastin degradation, preserved medial SMCs, and reduced number of infiltrating monocytes, macrophages, and T lymphocytes.

Among the Andro-sensitive chemokines/cytokines, CCL2, also called monocyte chemoattractant protein-1, is well known for its role in inflammatory recruitment in aneurysm. Targeted gene deletion of Ccl2 or its receptor Ccr2 confers aneurysm resistance (MacTaggart et al., 2007; Aoki et al., 2009; Moehle et al., 2011). In this study, Andro potently reduced the Ccl2 mRNA accumulation in the aortic wall by 93.18% compared with the solvent control. Andro also decreased the local expression of other established proinflammatory cytokines, including Tnf, Ifng, and Cxcl10. Inhibition of cytokine expression by Andro was replicated to a large extent in cultured aortic SMCs, as well as in a monocyte/macrophage cell line RAWs. Of note, inhibition of certain chemokines, such as Ccl2, Ccl7, and Cxcl10, was more profound in RAWs than in SMCs (80–95% vs. 25–30%). Since Andro produced a similar reduction in p65 phosphorylation in these two cell types, we speculate that the differential response reflects a cell-type-dependent transcription regulation of chemokines. Regulation of gene expression is a complex process requiring coordination of multiple transcription factors and coactivators, which is frequently controlled by epigenetic modifications. It is possible that NF-κB plays a

Fig. 3. Andro inhibits NF-κB activation and cytokine expression in TNFα-treated SMCs. (A and B) SMCs were pretreated with solvent (DMSO) or Andro (15 μM) for 1 hour before incubation with TNFα (10 ng/ml) for the indicated times. Whole-cell lysates were subjected to immunoblotting analysis with indicated antibodies. (C–I) SMCs were pretreated with solvent (DMSO) or Andro (15 μM) for 1 hour before incubation with TNFα (10 ng/ml) for 6 hours. mRNA expression of inflammatory chemokines and cytokines was analyzed by quantitative real-time PCR. All values represent the mean ± S.E.M. (n = 3–6; *P < 0.05, one-way analysis of variance (A–I). P-p65, phosphorylated p65.
more dominant regulatory role in chemokine transcription in monocytes/macrophages than in SMCs. However, this potential mechanism needs to be experimentally tested.

Our data revealed a novel function of Andro, i.e., inhibition of leukocyte adhesion. Aside from the reported inhibitory effect of Andro on expression of ICAM-1, VCAM-1, and E-selectin in ECs (Jiang et al., 2007; Wang et al., 2007; Lu et al., 2014), the present study demonstrated that Andro inhibited the expression of α4 integrin in monocytes/macrophages. To the best of our knowledge, this is the first demonstration that Andro inhibited the α4 integrin expression and endothelium adhesion of monocytes/macrophages. Interestingly, in the in vitro monocyte-endothelial cell interaction study, we found that pretreating RAWs with Andro (15 μM) produced a far more inhibitory effect on adhesion than pretreating endothelial cells.

In dose-response studies, Andro did not significantly decrease the viability of RAWs until it reached 30 μM. Preincubation of RAWs with 15 and 20 μM Andro reduced adhesion ability, but not viability, to the fibronectin-coated surface by 70 and 80%, respectively. However, differences in adhesion inhibition caused by these two doses of Andro did not reach significance, suggesting that Andro at 15 μM caused close-to-maximal inhibition of monocyte/macrophage adhesion. This observation is somewhat different from a prior study, which showed that 20 and 50 μM Andro dose-dependently suppressed NF-κB activity by 16.4 and 55.6%, respectively. However, neither concentration diminished cell viability, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Hsieh et al., 2011). It is possible that monocytes and macrophages are more sensitive to Ando or NF-κB inhibition. However, further experimental evidence is needed to confirm this possibility.

At the molecular level, we showed that Andro significantly decreased aortic activity of NF-κB. Whereas IkB degradation constitutes a canonical regulatory step for NF-κB activation (Oeckinghaus and Ghosh, 2009; Ren et al., 2014),

![Fig. 4. Andro inhibits NF-κB activation and cytokine expression in LPS-treated monocytes/macrophages. (A and B) RAW264.7 cells were pretreated with solvent (DMSO) or Andro (15 μM) for 1 hour before incubation with LPS (100 ng/ml) for the indicated times. Whole-cell lysates were subjected to immunoblotting analysis with indicated antibodies. (C–I) RAW264.7 cells were pretreated with solvent (DMSO) or Andro (15 μM) for 1 hour before incubation with LPS (100 ng/ml) for 6 hours. mRNA expression of inflammatory chemokines and cytokines was analyzed by quantitative real-time PCR. All values represent the mean ± S.E.M. [n = 4–5; *P < 0.05, one-way analysis of variance (A–F and H) and two-tailed Student's t test (G and I)]. P-p65, phosphorylated p65.](image-url)
post-translational modifications of p65, such as phosphorylation, influence its dimerization, DNA binding, and transcriptional activity, and are recognized as an alternative mechanism leading to NF-κB activation (Oeckinghaus and Ghosh, 2009). The effect of Andro on NF-κB activation appears to depend on the cell type and stimulus. In collagen-stimulated platelets, Andro affects NF-κB at IκB degradation (Lu et al., 2012). In SMCs, Hsieh et al. reported that Andro suppressed LPS/interferon-γ–induced p65 nuclear translocation, p65 phosphorylation, and DNA binding activity, but not degradation of IκB (Hsieh et al., 2011). Consistent with their findings, we found that Andro decreased p65 phosphorylation at Ser536 in both TNFα-treated SMCs and LPS-treated RAWs. However, Andro did not significantly alter IκB degradation in either cell type. We speculate that this IκB-independent effect of Andro allows cells to maintain certain NF-κB–mediated antiapoptotic proteins, including Bcl-2, A1, and IAP. This notion may explain the low cytotoxicity of Andro that we and other groups observed (Xia et al., 2004; Wang et al., 2007; Hsieh et al., 2011).

The involvement of NF-κB in aneurysm pathophysiology is implicated by a markedly elevated NF-κB activity in biopsies of human aneurysmal tissues and a mouse model of AAA (Miyake et al., 2006, 2007). Using an antibody specific to the
activated p65, we confirmed the prior reports and identified SMCs and macrophages as major cell types that harbor activated NF-κB. At 14 days post aneurysm induction, CD31-positive ECs remained sporadic in the elastase-treated aortic sections, likely due to endothelial damage during the perfusion procedure. The contribution of endothelial-specific NF-κB activation in aneurysm has been previously highlighted. Using a transgenic mouse line expressing dominant-negative IκBα selectively in ECs, Saito et al. (2013) demonstrated that selective NF-κB inhibition decreased macrophage infiltration and aneurysmal dilatation in the angiotensin II AAA model. The involvement of NF-κB in aneurysm development was also demonstrated by Nakashima et al. (2004), who inhibited the signaling pathway with an oligodeoxynucleotide decoy delivered to the aortic wall during the elastase perfusion procedure in rats. Given the established effects of Andro on the NF-κB pathway and the involvement of NF-κB in inflammation and aneurysm pathophysiology, it is plausible to attribute the antianeurysm effect of Andro to NF-κB inhibition. However, our data do not exclude other potential targets.

Beneficial effects of Andro have also been observed in several other experimental cardiovascular disease models, including restenosis, myocardial infarction, and hyperglycemia (Yu et al., 2003; Wang et al., 2007; Woo et al., 2008). Although these prior studies and our current work put Andro in a strong position as a drug candidate, caution should be taken when translating mouse work into clinical treatments. One concern relates to aneurysm modeling. Several murine

![Fig. 6. Andro inhibits inflammatory cytokine expression in elastase-perfused aortic tissues. mRNA expression of Ccl2 (A), Cxcl10 (B), Tnf (C), Ifng (D), Ccl5 (E), Ccl7 (F), and Cxcl16 (G) in aorta of solvent- or Andro-treated animals 14 days after surgery was analyzed by quantitative real-time PCR. All values represent the mean ± S.E.M. (n = 3–8; *P < 0.05, one-way analysis of variance). E, elastase; IE, inactivated elastase.](image)

![Fig. 7. Andro decreases infiltration of inflammatory cells in elastase-perfused aortic tissues. (A) Representative images of arterial sections immunohistochemically stained for MOMA-2 (a monocyte and macrophage marker), CD3 (a T cell marker), and Ly6G (a neutrophil marker). Higher-magnification views of boxed areas are shown at the lower-left corner. E, elastase. Scale bar = 200 μm. (B) Quantification of inflammatory cell infiltration in aortic tissue is expressed as positive cells/nuclei. All values represent the mean ± S.E.M. (n = 6–10; *P < 0.05, two-tailed Student’s t test).](image)
AAA models have been created with different strengths and weaknesses (Daugherty and Cassis, 2004). In this study, we used an elastase-induced AAA model, which recapitulates important pathologic features of human AAA, including SMC depletion, inflammation, and elastin degradation (Daugherty and Cassis, 2004; Thompson et al., 2006). However, it does not lead to AAA rupture, a major complication in human AAA (Nordon et al., 2011). Another concern in translation is drug toxicity. In this study, the mortality rate was 0 in both Andro-treated (n = 7) and solvent-treated (n = 9) groups. Consistent with prior studies, no differences in body weight and appearance were noticed between Andro- and solvent-treated mice. The safety of Andro is supported by a phase II clinical study of rheumatoid arthritis patients, which revealed that A. paniculata tablets containing 30 mg of andrographolide (three times a day for 14 weeks) were well tolerated and significantly more effective in reducing symptoms and serological parameters of the disease (Burgos et al., 2009). However, higher doses of Andro (4–6 mg/kg body weight, oral administration) may cause isolated cases of allergic reactions, tiredness, headache, pruritus/rash, diarrhea, nausea, metallic taste, bitter taste, and dry tongue (Coon and Ernst, 2004). Three additional clinical trials are currently being conducted to evaluate efficacy, safety, and tolerability of Andro in patients with multiple sclerosis and colorectal neoplasms.

In closing, our results indicate that Andro suppresses small aneurysm progression in an elastase-induced AAA mouse model. The therapeutic effect of Andro on AAA progression is achieved at least in part through inhibition of NF-κB activity, which subsequently diminishes production of inflammatory cytokines and chemokines as well as integrins. Thus, Andro may offer a safe and effective therapeutic strategy to slow disease progression in patients with small AAAs.

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Authorship Contributions

Participated in research design: Ren, Z. Liu, Wang, B. Liu.
Conducted experiments: Ren, Z. Liu, Wang, Giles, Greenberg.
Contributed new reagents or analytic tools: Sheibani.
Wrote or contributed to the writing of the manuscript: Ren, Sheibani, Kent, B. Liu.

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Address correspondence to: Dr. Bo Liu, Department of Surgery, University of Wisconsin–Madison, 1111 Highland Avenue, WIMR 5137, Madison, WI 53705. E-mail: liub@surgery.wisc.edu