A Modified Sesamol Derivative Inhibits Progression of Atherosclerosis

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Objective—Sesamol, a phenolic component of lignans, has been previously shown to reduce lipopolysaccharide-induced oxidative stress and upregulate phosphatidylinositol 3-kinase/Akt/endothelial nitric oxide synthase pathways. In the present study, we synthesized a modified form of sesamol (INV-403) to enhance its properties and assessed its effects on atherosclerosis.

Methods and Results—Watanabe heritable hyperlipidemic rabbits were fed with high-cholesterol chow for 6 weeks and then randomized to receive high-cholesterol diet either alone or combined with INV-403 (20 mg/kg per day) for 12 weeks. Serial MRI analysis demonstrated that INV-403 rapidly reduced atherosclerotic plaques (within 6 weeks), with confirmatory morphological analysis at 12 weeks posttreatment revealing reduced atherosclerosis paralleled by reduction in lipid and inflammatory cell content. Consistent with its effect on atherosclerosis, INV-403 improved vascular function (decreased constriction to angiotensin II and increased relaxation to acetylcholine), reduced systemic and plaque oxidative stress, and inhibited nuclear factor–κB activation via effects on nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) phosphorylation with coordinate reduction in key endothelial adhesion molecules. In vitro experiments in cultured endothelial cells revealed effects of INV-403 in reducing IκBα phosphorylation via inhibition of IκB kinase 2 (IKK2).

Conclusion—INV-403 is a novel modified lignan derivative that potently inhibits atherosclerosis progression via its effects on IKK2 and nuclear factor–κB signaling. (Arterioscler Thromb Vasc Biol. 2011;31:536-542.)

Key Words: atherosclerosis ■ macrophages ■ vascular biology

Atherosclerosis is a complex, multifactorial disease that involves distinct stages that accrue over a lifetime.1,2 Oxidative stress and inflammation play a fundamental role in the genesis of all of these processes3-5 and represent targets for intervention. Current approved therapies for the prevention and treatment of atherosclerosis suffer from important drawbacks, including the inability to address multiple mechanisms involved in progression in atherosclerosis and side effects, especially those related to unrecognized off-target effects, when targeting highly specific pathways.7,8

Dietary approaches are inherently powerful as they are safe and can be initiated early and over the long term for an individual, providing for the greatest likelihood for success.9-17 A number of dietary strategies have the capability of simultaneously addressing distinct pathophysiologically relevant processes.18-25 Successful implementation, however, warrants complete understanding of the effects of the intervention on these pathways.19 In addition, no single dietary intervention combines all aspects of an ideal intervention.17,18,26-32 Thus, fortification of existing dietary regimens with simple and safe components derived from other regimens may be preferable.17,18 In many Asian cultures, ingredients used in cooking have been implicated in health benefits over thousands of years.34 Oil seeds, such as flaxseeds and soybeans, are well-known repositories of small molecule chemical components that include isoflavones, sterols, tocopherols, tocotrienols, and lignans.23,35-37 Sesamol, a phenolic component of lignan derivatives, such as sesamin and sesaminol, is generated upon roasting of sesame seeds and during the bleaching process of sesame oil, and it has previously been shown to reduce lipopolysaccharide-induced oxidative stress38 and upregulate phosphatidylinositol 3-kinase/Akt/endothelial nitric oxide synthase pathways.39 In the present study, we synthesized a modified form of sesamol (INV-403) and demonstrated that this small molecule markedly inhibited atherosclerosis in a rabbit model, paralleled by a decrease in aortic inflammation oxidative stress and improvement in endothelial function, mediated in part by its effects on inhibition of IKK2 and consequent reduction in nuclear factor–κB (NF-κB) activation without overt changes.
Methods

Animal Model

The Institutional Animal Care and Use Committee at the Ohio State University approved the experimental animal protocols. Ten male WHHL rabbits (2 months old) were obtained from the Brown family in Odenville, Alabama and allowed to acclimate for 2 weeks before being fed with high-cholesterol chow (fat: 2.7%, wt/wt; cholesterol: 0.5%, wt/wt; Harlan Teklad TD87251) for 6 weeks, at which time point they were randomly assigned to control or INV-403 groups (20 mg/kg per day for 12 weeks). INV-403 was dissolved in 90% ethanol and sprayed onto the high-cholesterol chow. The diet-drug mixture was then vacuum dried overnight to remove ethanol and then used to feed rabbits.

Other Methods

Other methods are described in the Supplemental Methods, available online at http://atvb.ahajournals.org.

Statistics

Results are expressed as means±SD. The unpaired Student t test was used to compare parameters in the INV-403 and control treated groups. Probability values <0.05 were reported as significant. With multiple comparisons, a Bonferroni correction was used for multiple comparisons. In vitro experiments comparing INV-403 with other antioxidants involving multiple groups were analyzed using 1-way ANOVA with a Bonferroni post hoc correction.

Results

We first assessed the effect of INV-403 on atherosclerosis in a rabbit model as illustrated in Figure 1A. To enhance the atherosclerosis progression, all rabbits were fed with high-cholesterol chow for a period of 6 weeks before being randomly assigned to the control or INV-403 group. Dietary supplementation with INV-403 started at the end of 6 weeks for a duration of 12 weeks. This dietary supplementation with INV-403 did not significantly affect rabbit weight (control versus INV-403: 3.16±0.13 versus 3.16±0.08 kg) or the level of any of the plasma lipoprotein subfractions (Supplemental Figure I).

The wall volume in the rabbit abdominal aorta, an indicator of atherosclerotic plaque, was analyzed by serial in vivo MRI scanning. Figure 1B and 1C shows that after 6 weeks of dietary supplementation with INV-403, wall volume in the abdominal aorta was significantly reduced compared with that in the control arm. At the end of 12 weeks, there was a more pronounced slowing of the rate of progression of plaque in the INV-403 group (Figure 1B and 1C). Morphometric assessment of plaque burden in the thoracic aorta at the end of 12 weeks corroborated the antiatherosclerotic effects of INV-403 on the abdominal aorta (Figure 1D and 1E). Immunohistochemical analysis of thoracic aortic sections revealed a reduction in CD68+ cells in atherosclerotic plaque but increased smooth muscle proliferation (Figure 2A and 2B). INV-403 also decreased lipid accumulation and reparative fibrillar collagen (Figure 2C and 2D) in the rabbit aorta.

Figure 3 and Supplemental Figure II and the Table illustrate the responses to various agonists in the thoracic aorta of WHHL rabbits. Figure 3A and 3B depicts the effects of INV-403 on vascular function in response to classical agonists of endothelial and smooth muscle function. INV-403 increased relaxation to acetylcholine, whereas aortic constriction to angiotensin II was diminished. In contrast to these effects, INV-403 had no effects on responses to insulin (partial endothelium dependent agonist) or sodium nitroprusside (SNP, an endothelium independent vasodilator) (Supplemental Figure II) and had no effects on constriction in plasma lipid profile in a Watanabe heritable hyperlipidemic (WHHL) model.

Table. The Maximal Effect and Log EC50 of Aortic Responses

<table>
<thead>
<tr>
<th></th>
<th>PE Maximal</th>
<th>Log EC50 Maximal</th>
<th>ET-1 Maximal</th>
<th>Log EC50 Maximal</th>
<th>All Maximal</th>
<th>Log EC50 Maximal</th>
<th>Ach Maximal</th>
<th>Log EC50 Maximal</th>
<th>SNP Maximal</th>
<th>Log EC50 Maximal</th>
<th>Insulin Maximal</th>
<th>Log EC50 Maximal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.2±6.5</td>
<td>6.6±0.1</td>
<td>52.3±9.5</td>
<td>7.3±0.3</td>
<td>19.8±1.2</td>
<td>8.1±0.1</td>
<td>27±4</td>
<td>5.7±0.2</td>
<td>80.8±6.7</td>
<td>6.6±0.2</td>
<td>69.3±10.7</td>
<td>3.7±1.2</td>
</tr>
<tr>
<td>INV-403</td>
<td>135.4±5.1</td>
<td>6.7±0.1</td>
<td>47.3±9.1</td>
<td>7.5±0.4</td>
<td>12.6±1.1</td>
<td>8±0.2</td>
<td>73.6±2.3</td>
<td>6.3±0.1</td>
<td>72±5.9</td>
<td>6.8±0.1</td>
<td>71±9.9</td>
<td>4.2±0.7</td>
</tr>
</tbody>
</table>

Maximal effects are expressed as % of contraction by KCl (120 mM). PE, phenylephrine; ET-1, endothelin-1; All, angiotensin II; Ach, Acetylcholine; SNP, sodium nitroprusside.

*P<0.05, two way ANOVA.
response to phycoerythrin (PE) and endothelin-1. The attenuated response to angiotensin II in INV-403 rabbits was paralleled by decreased mRNA expression of angiotensin II receptor, type 1 (AT1) receptor (Figure 3C). In contrast, treatment with INV-403 did not change the expression of AT2 receptor (Figure 3C).

Figure 4A and 4B reveals the effect of INV-403 in reducing oxidative stress markers in plasma of animals and in aortic homogenates respectively. These results were confirmed by in situ aortic \( \text{O}_2^\cdot \) measurement using the dihydroethidium staining (Figure 4C and 4D). As phenolic antioxidants have been thought to exert direct antioxidant effects, we tested the effects of INV-403 on superoxide generation in response to NADPH stimulation. INV-403 (up to 1 mmol/L) did not reduce superoxide production on the addition of NADPH, indicating that INV-403 decreases oxidative stress in vivo through mechanisms other than direct scavenging of reactive oxide species (data not shown).

As vascular inflammation plays a pivotal role in atherosclerosis, we assessed proinflammatory gene expression and cellular correlates of inflammation. INV-403 decreased mRNA expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1, monocyte chemoattractant protein-1 (MCP-1), and L-selectin in thoracic aorta (Figure 5A). The mRNA expression of P-selectin showed a nonsignificant decrease in the INV-403 group (Figure 5A). Macrophage infiltration was decreased as evidenced by immunohistochemistry (Figure 2B) and analysis of CD68 gene expression (Figure 5B). VCAM-1 is expressed in various types of cells, including endothelial cells, smooth muscle cells, and macrophages. Figure 5C reveals that INV-403 treatment decreased VCAM-1 throughout the plaque, indicating that INV-403 may reduce VCAM-1 expression in plaque via both reduced macrophage infiltration and attenuated expression of VCAM-1 expression in other cell types. We corroborated the effect of INV-403 on VCAM-1 and intercellular adhesion molecule-1 expression by measuring expression of these adhesion molecules at the protein level (Figure 5D and 5E). Because NF-\( \kappa \)B is a critical transcription factor regulating the expression of proinflammatory genes, such as VCAM-1, we examined the effect of INV-403 on NF-\( \kappa \)B activity. As shown in Figure 5F and 5G, INV-403 markedly decreased the DNA binding activity of NF-\( \kappa \)B.

Given that NF-\( \kappa \)B activation can be inhibited by antioxidants and INV-403 is a derivative of sesamol, which is an antioxidant in vitro, we examined whether INV-403 inhibits
NF-κB dependent on its antioxidant effects. Figure 6A demonstrates that in contrast to sesamol, which scavenged superoxide induced by NADPH in liver lysates, INV-403 did not exert antioxidant effects, indicating that INV-403 inhibits NF-κB by antioxidant-independent mechanisms. To assess the mechanism by which INV-403 inhibits NF-κB, bovine aortic endothelium cells (BAECs) were transfected with NF-κB reporter construct and then treated with INV-403.

Results show that INV-403 dose-dependently decreased tumor necrosis factor-α-induced NF-κB-mediated luciferase expression, whereas sesamol did not inhibit TNF-α-induced luciferase expression (Figure 6B). We corroborated the effect of INV-403 on NF-κB signaling by measuring the dose-dependent inhibition of TNF-α-induced VCAM-1 expression in BAECs (Figure 6C). In contrast, sesamol did not inhibit VCAM-1 expression (Figure 6D). NO is a potent inhibitor of NF-κB signaling and sesamol can induce NO production. We therefore tested whether the effect of INV-403 on NF-κB signaling was mediated by NO production. Figure 6E and 6F show that a NOS inhibitor, L-NAME, did not inhibit the effect of INV-403 on NF-κB mediated luciferase and VCAM-1 expression in BAECs, further supporting that the inhibition of NF-κB by INV-403 is not mediated by NO. We also tested the effects of a nonspecific antioxidant N-acetyl-L-cysteine (NAC) and compared its effects with INV-403. NAC decreased TNF-α-induced NF-κB activation, but not to the extent seen with INV-403. We did not observe significant effects of NAC on TNF-α-induced VCAM-1 expression (Figure 6E and 6F).

To determine the target of INV-403 for the inhibition of NF-κB signaling, we examined the effect of INV-403 on the TNF-α-induced IκBα phosphorylation and subsequent degradation, a mechanism central to the activation of NF-κB. Figure 6G depicts the effect of INV-403 on IκBα. INV-403...
markedly reduced TNF-α-induced IκBα phosphorylation and degradation in BAECs. Because the ubiquitin-proteasome system is involved in the degradation of IκB and may serve as a mechanism by which certain drugs may modulate NF-κB activation, we tested the effects of a classic proteasomal inhibitor MG-132 alone or when used in conjunction with INV-403. As anticipated, MG-132 treatment markedly reduced IκBα degradation without effects on phosphorylation of IκB. However when MG-132 was used in conjunction with INV-403, in addition to the expected reduction in IκBα degradation, there was an effect on IκBα phosphorylation. These results suggest a specific effect of INV-403 on IKK2-mediated phosphorylation rather than an effect on proteasomal degradation of IκBα.

We then tested the effects of INV-403 on IKK2 using an in vitro kinase assay. Figure 6H demonstrates that whereas sesamol almost have no effect on IKK2 activity, INV-403 dose-dependently inhibited IKK2 in vitro, supporting our in vivo data that INV-403 may inhibit NF-κB activation through IKK2-dependent mechanisms.

Discussion

The present study has multiple findings. (1) Dietary supplementation with a simple modified lignan derivative for a 12-week period significantly decreased atherosclerotic plaque burden, with changes seen as early as 6 weeks without effects on lipid profile in the WHHL model. (2) Decreased plaque burden was paralleled by marked improvements in endothelial function, vasoconstrictor responses (decreased response to angiotensin II with reduced AT1R expression) and inflammation. (3) There was inhibition of plaque NF-κB signaling through targeting IKK2. A, In the presence of the indicated compounds, NADPH-induced superoxide production in mouse liver lysate was analyzed with lucigenin. n=3; \( P<0.05 \) versus NADPH only; \( P<0.05 \) versus negative control; ANOVA. B, After transfection with pGL2/NF-κB-Luc, BAECs were pretreated with the indicated concentration of INV-403 or sesamol for 30 minutes and then stimulated with TNF-α (10 ng/mL) for 4 hours, and the activity of luciferase was assessed. A summary of 3 independent experiments is presented. * \( P<0.05 \) versus TNF-α only; 1-way ANOVA. C, BAECs were pretreated with the indicated concentration of INV-403 or vehicle for 30 minutes and then stimulated with TNF-α (10 ng/mL) for 6 hours. The expression of VCAM-1 was assessed by Western blot analysis. A representative image and the summary of 4 independent experiments are presented. D, BAECs were pretreated with vehicle, INV-403 (100 μmol/L), or sesamol (100 μmol/L) for 30 minutes and then stimulated with TNF-α (10 ng/mL) for 6 hours. The expression of VCAM-1 was assessed by Western blot analysis. A representative image and the summary of 4 independent experiments are presented. E, BAECs were transfected with pGL2/NF-κB-Luc and then treated with the indicated compounds. The luciferase activity was then analyzed. n=4; \( P<0.05 \) versus negative control; \( P<0.05 \) versus TNF-α only; ANOVA. F, BAECs were pretreated with the indicated compounds for 30 minutes and then stimulated with TNF-α (10 ng/mL) for 6 hours. The expression of VCAM-1 was assessed by Western blot analysis. A representative image and the summary of 4 independent experiments is presented. H, The dose-dependent effect of INV-403 and sesamol on IKK2 activity in vitro as assessed with HTScan IKKβ Kinase Assay Kit (Cell Signaling Technology).
in BAECs, which showed that INV-403 inhibited TNF-α-induced NF-κB-dependent luciferase expression and VCAM-1 expression. NF-κB activation is initiated by IκB phosphorylation by IKK and its subsequent degradation through the ubiquitin-proteasome system. INV-403 markedly inhibited TNF-α-induced IκBα degradation in BAECs, which appeared to result from decreased IκBα phosphorylation without effects on proteasomal degradation, because its effects did not mimic those of MG-132, a classic proteasomal inhibitor. These results, in conjunction with the inhibitory effects of INV-403 on IKK2, suggest that this is the predominant pathway by which NF-κB activation is inhibited. Notably, although it has been demonstrated that NF-κB inhibitors can reduce atherosclerotic lesion and IKK2 is critical for activation of NF-κB, the effects of specific IKK2 inhibitors on atherosclerosis has not yet been assessed. INV-403 markedly decreases inhibited macrophage infiltration in plaque and was also associated with a reduction in reparative collagen. These changes were associated with potent effects in improving endothelial function. The role of NF-κB in atherosclerosis is complex with a differential cell-specific role for this pathway, which may also differ at various stages of atherosclerosis. In keeping with this concept, cell-specific conditional ablation of this pathway early in atherosclerosis has a favorable effect. In contrast, the NF-κB pathway may play an alternate protective role in macrophage cells, with disruption of this pathway resulting in progression of atherosclerosis. These contrasting effects suggest that any pharmacotherapeutic approach that modulates NF-κB may need to reconcile contrasting roles of this entity. Our data do not provide cell-specific effects of INV-403, and this is a limitation of our data.

INV-403 reduced constriction to angiotensin II but had no effects on endothelin-1 or phenylephrine-mediated constriction. This occurred through downregulation of AT-1 mRNA expression in the aorta in response to treatment with INV-403. The renin-angiotensin-aldosterone system, a crucial regulator of vascular homeostasis, has been consistently shown to play a crucial role in atherosclerosis. Conversely, blockade of AT1 receptors in atherosclerosis normalizes oxidative stress, improves endothelial function, and reduces plaque area and macrophage infiltration. Thus, INV-403 may potentially exert additional synergestic effects with drugs that target the renin-angiotensin system (RAAS) system in atherosclerosis, a finding that may be worthy of exploration in the future. Notably, these effects occurred in the absence of any changes in plasma lipid profile in the fat-fed WHHL rabbit.

Our novel serial MRI scanning protocol allowed assessment of the time course of effects and showed that 6 weeks of dietary supplementation with INV-403 was sufficient to inhibit atherosclerotic lesion development in WHHL rabbits. The assessment of abdominal aortic atherosclerosis by MRI also served as an adjunctive measure of morphometric quantification of atherosclerosis in the thoracic aorta and confirmed effects of the drug in both these territories. Such an noninvasive approach may be helpful in assessment of time course of pharmacotherapies in rabbit models as it involved usage of a clinically relevant field strength (1.5 T) and does not require more expensive high-field MRI systems.

Notably, no significant changes in plasma lipoprotein levels were observed by the end of the 12 weeks of dietary supplementation with INV-403. In prior studies, we have demonstrated an important effect of sesame oil in reducing atherosclerosis in conjunction with reduction in plasma lipoprotein components in an low-density lipoprotein receptor (−/−) model. In this prior work, we postulated that non-saponifiable components of sesame oil such as sesamin and sesamolin may be worth exploration in the development of modified lignan component alone (INV-403). These findings may suggest unique effects of individual or modified components that may be distinct from that of sesame oil. Sesame oil is rich in both polyunsaturated fatty acids and mono-unsaturated fatty acids (approximately 47% oleic acid and 39% linoleic acid) and also contains lignans, such as sesamin and sesamolin, and several antioxidant compounds, such as sesaminol. Sesamin feeding is associated with a reduction of serum lipid levels in rodents, concomitant with an increased fatty acid oxidation. This effect has been attributed to the ability of sesamin to affect peroxisome proliferator-activated receptor (PPAR)–mediated transcriptional events. Activation of PPAR has been demonstrated to modulate many aspects of lipoprotein metabolism and inflammation in vitro, as well as in animal and human studies. In our studies, we did not see in vivo effects of INV-403 consistent with PPAR-α activation (reduction in plasma triglycerides or increase in high-density lipoprotein) or increase in acyl-CoA oxidase expression in the liver (data not shown). Moreover the drug did not activate PPAR-γ or PPAR-Δ in HepG2 cells using the yeast UAS-TK, Gal4 system (data not shown). In addition, it may be that the extremely high levels of non–high-density lipoprotein cholesterol in the WHHL model (related to mutations in low-density lipoprotein receptor), especially in response to high-fat chow, may render any effects on plasma lipoprotein profile indiscernible. Further studies in milder models of hyperlipidemia, preferably not involving genetic manipulation of lipoprotein clearance (eg, hamster model of diet induced hyperlipidemia) may be required to tease out the effects on plasma lipid profile.

INV-403 was synthesized from 3,4-methylenedioxyphenol, via synthetic modification to allow enhanced stability of the phenolic group and to allow NO release. Our data, however, indicate that the introduced nitro group did not directly influence acute NO release. The nitro group was nevertheless important in mediating the effects of the drug, such as the induction of low-density lipoprotein receptor in HepG2 cells. This was confirmed via structure-activity relationship analysis (data not shown).

In conclusion, INV-403 exerts important vascular protective effects in a model of severe hyperlipidemia atherosclerosis. These findings may have implications for further testing of modified forms of sesame lignans in the treatment of atherosclerosis.

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Disclosures

Intellectual property in this work belongs to Ohio State University and is licensed to InVasc Therapeutics.

References


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Supplementary Methods

Methods:
The Institutional Animal Care and Use Committee (IACUC) at The Ohio State University approved the experimental animal protocols.

**Synthesis of 3,4-methylenedioxy-6-nitrophenyl acetate**
The rationale for simple chemical modification on 3,4-methylenedioxy phenol is to provide additional therapeutic benefits by way of introducing acetyl group at phenolic OH and nitro group at the 6th position. The acetyl group was introduced with the intent of providing chemical stability to sesamol by preventing aerial oxidation rendering longer shelf life. The nitro group was added to provide additional "NO" release functionality in-vivo. The synthesis of INV 403 is achieved in two step process starting from 3,4-methylenedioxyphenol which is schematically represented below:

Acetylation of 3,4-methylenedioxy phenol using fresh sample of acetic anhydride in presence of 10% NaOH yielded 3,4-methylenedioxyphenyl acetate in 75% chemical yield. The nitration of 3,4-methylenedioxyphenyl acetate using concentrated nitric acid in presence of glacial acetic acid provided 3,4-methylenedioxy-6-nitro phenyl acetate in 70% yield as a pale yellow solid. The final compound was further purified by recrystallization from 95% ethanol to get crystalline yellow material.

**Animal Models of Atherosclerosis**

*Rabbit Model of Atherosclerosis*: Ten male Watanabe Heritable Hyperlipidemic WHHL rabbits (WHHL, 2 months old) were obtained from the Brown Family in Alabama and allowed to acclimate for 2 weeks prior to being fed with high cholesterol chow (fat: 2.7% wt/wt; cholesterol: 0.5% wt/wt; Harlan Teklad TD87251) for 6 weeks, at which time point they were randomized to control or INV-403 groups (20 mg/kg/d for 12 weeks).
INV-403 was dissolved in 90% ethanol and sprayed onto the high cholesterol chow. The diet-drug mixture was then vacuum dried overnight to remove ethanol and then used to feed rabbits. The dose of 20 mg/kg was selected according to published data [Food Chem Toxicol. 2008 46(8):2736-41; Shock. 2008;30(4):456-62.], which indicated that around 10 mg/kg sesamol was safe and effective. Considering the different molecular weight (224.15 for INV-403 and 138.12 for sesamol), 20 mg/kg INV-403 was used to treat animals.

**In vivo Magnetic Resonance Imaging (MRI) to Assess Atherosclerosis Progression**

Atherosclerotic plaque in the abdominal aorta of WHHL rabbits was analyzed by in vivo MRI scans using a 1.5T Siemens clinical scanner at the indicated time points. Kidneys were used as anatomical landmarks and were depicted using a coronal gradient echo, T1-weighted localizing sequence (TR/TE 800/1.0). Forty-eight, axial slices spanning the iliac bifurcation to the superior pole of the topmost kidney were obtained using a T1-weighted gradient echo turbo FLASH protocol (FOV 288/384; TR/TE 230/5.6; NEX=3; BW 100MHz; time of acquisition of ~11 minutes). No respiratory or cardiac gating was necessary as the abdominal aorta is relatively free from motion artifact. Plaque burden was determined by manually tracing the external elastic lamina (EEM) and the luminal border (L) and determining the area within each boundary using Siemens Viewer software. Slice volume (V) was calculated as \( V = (\text{EEM-L}) \times 4.0 \) and total wall volume per animal was calculated using the formula \( \text{TWV} = \Sigma [(\text{EEM-L}) \times 4] \) and expressed in \( \text{mm}^3 \). TWVs for each animal at each time point were normalized for varying numbers of readable slices with the formula \( \text{NWV} = (\text{TWV}/n) \times m \); \( n= \text{number of slices readable in the individual animal and } m= \text{mean number of slices readable in all animals over all time points. All normalized wall volumes were reported in } \text{mm}^3 \).

**Immunohistochemistry and Morphometry**
Supplementary Methods

Analysis of Atherosclerosis: Segments of descending thoracic aorta were embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek USA Inc, Torrance, Calif) and frozen on dry ice. En face sections were then prepared. To analyze atherosclerotic burden, 8-12 sections (4 µm thick) were collected at intervals of 20 µm. After H&E staining and Oil-red O staining, each section was analyzed in a blinded manner after digitizing the images. The images were analyzed under a research microscope (Zeiss Axioskop with Spot I digital camera, Jena, Germany) with National Institutes of Health (NIH) Image software version 1.61 (Wayne Rasband, NIH, http://rsb.info.nih.gov/nih-image). Results were expressed as mm².

Immunohistochemistry: Immunohistochemical staining was performed by using the primary antibodies and a detection system (Immunoperoxidase Secondary Detection System; Chemicon International, Temecula, Calif), and quantified with software (NIH Image) after digitization of the images with a camera system (Zeiss Axioskop with Spot I digital camera). Antibodies against CD68 were purchased from Santa Cruz Biotechnology Incorporated (Santa Cruz, Calif). A polyclonal anti-α-Actin antibody was obtained from Upstate Cell Signaling Solutions (Lake Placid, NY). T cell receptor β antibody was obtained from Biolegend (San Diego, CA). Anti-VCAM1 antibody was bought from R & D systems. To quantify the staining, the mean density of 4 negative control (no primary antibody) sections was set as the threshold, and positive area was acquired by software. Results were finally normalized by the area subtended by the external elastic lamina to the luminal interface or the intimal-medial volume.

Vascular Physiology Studies

Rabbits were euthanized by injection of lethal doses of pentobarbital. The ascending aortas was removed and 3 mm thoracic aortic rings were suspended in individual organ chambers filled with physiological salt solution buffer (sodium chloride, 130 mEq/L; potassium chloride, 4.7 mEq/L; calcium dichloride, 1.6 mEq/L; magnesium sulfate, 1.17
Supplementary Methods

mEq/L; potassium diphosphate, 1.18 mEq/L; sodium bicarbonate, 14.9 mEq/L; EDTA, 0.026 mEq/L; and glucose, 99.1 mg/dL [5.5 mmol/L]; pH, 7.4), aerated continuously with 5% carbon dioxide in oxygen at 37°C. Vessels were allowed to equilibrate for 1 hour at a resting tension of 30 mN before being subjected to graded doses of agonists as described previously\(^1\), \(^2\). The vasoconstrictor agonists included phenylephrine (PE), endothelin-1 (ET-1), or angiotensin II. Responses were expressed as a percentage of the peak response to 120 mEq/L of potassium chloride. The vessels subjected to PE were washed thoroughly and allowed to equilibrate for 1 hour before beginning experiments with acetylcholine or SNP. After a stable contraction plateau was reached with PE (0.1 \(\mu\)M), the rings were exposed to graded doses of the endothelium-dependent agonist acetylcholine or the endothelium-independent agonist SNP. Results were expressed as a percentage of pre-contraction by PE (0.1 \(\mu\)M). The rings exposed to acetylcholine were thoroughly washed and allowed to equilibrate for 1 hour. After a stable contraction plateau was reached with PE (0.1 \(\mu\)M), insulin was then added in an accumulative manner. Results were expressed as a percentage of pre-contraction by PE (0.1 \(\mu\)M).

**In-situ Detection of O\(_2^\cdot\)**

Briefly, in situ detection of O\(_2^\cdot\) was performed in snap-frozen aortic tissues embedded in OCT compound (Tissue-Tek®, Sakura Finetek USA Inc, Torrance, CA) as previously described.\(^3\) Tissue samples were cryosectioned at 4 \(\mu\)m of thickness, collected onto Superfrost Plus slides (Fisher Scientific, Pittsburg, PA), and stored at -80°C until needed. Four slides that were randomly chosen from each rat (tissue block) were placed into phosphate buffered saline (PBS) for 30 min at room temperature and then stained with dihydroethidium (DHE, 10 \(\mu\)M, Molecular Probes, Inc., Eugene, OR) in PBS for 20 min in a moist chamber in the dark. The slides were rinsed extensively with PBS,
Supplementary Methods

coverslipped, and digitally imaged with a research microscope (Zeiss Axioskop with a Spot I digital camera, Jena, Germany). To quantify the staining, the mean density of the area subtended by the external elastic lamina to the luminal interface or the intimal-medial volume is acquired.

8-epi Prostaglandin F2α (8-Isoprostane) Enzyme Linked Immunoassay

Stat-8-Isoprostane ELISA kit (Cayman Chemical, , Ann Arbor, Mich) was used to determine 8-isoprostane level in plasma and liver. Both samples were prepared according to the manufacturer's instruction, and underwent affinity purification (Cayman Chemical, Ann Arbor, Mich). The 8-isoprostane levels in the liver were adjusted by protein amount, which were determined by BCA Protein Assay (Pierce, Rockford, IL.), while 8-isoprostane levels in the plasma were normalized by the starting volume of plasma.

Western Blot Analysis

Samples were homogenized and solubilized in radioimmunoprecipitation assay buffer (RIPA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride, with 0.25% sodiumdeoxycholate and 1.0% Nonidet P-40) and centrifuged at 10,000g and 4°C for 30 min. The supernatant was collected and subjected to western blot analysis. In brief, 40 µg of protein was separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membrane. The membrane was then incubated with: mouse anti-β-actin (Sigma, St. Louis, MO), mouse anti-VCAM1, monoclonal anti-ICAM1 (R&D Systems, Minneapolis, MN), mouse anti-phospho-IκBα(Ser32/36), rabbit anti-IκBα (Cell Signaling Technology, Danvers, MA). Finally, the membranes were incubated with a horseradish peroxidase-linked secondary antibody and visualized with an enhanced
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chemiluminescence kit (Amersham Biosciences Inc., Piscataway, NJ). Band density was quantified by densitometric analysis using ImageJ.

**NF-κB Activation Assays**

Nuclear proteins were extracted from rabbit aorta with NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL) and subject to electrophoretic mobility shift assay (EMSA) with lightshift Chemiluminescent EMSA Kit (PIERCE, Rockford, IL) according to the manufacturer’s instruction. Oligonucleotides (Sequences: NF-kappaB sense, AgT TgA ggg gAC TTT CC Cag gC; NF-kappaB antisense, gCC Tgg gAA AgT CCC CTC AAC T) were synthesized by Invitrogen, and labeled with Biotin 3’ End DNA labeling Kit (PIERCE, Rockford, IL). Binding Reactions were performed in 20 µl solution containing 10x binding buffer (2 µl), Glycerol (2.5%), MgCl₂ (5 mM), Poly(dI·dC) (50 ng/µl), NP-40 (0.05%), Nuclear extract (8 µg proteins), and Biotin-DNA (20 fmol). The binding action products were then resolved by 6% native polyacrylamide gel and transferred to nylon membrane. After cross-linking, biotin–DNA was visualized by chemiluminescence.

**IKK2 Activity Assay**

HTScan® IKKβ Kinase Assay Kit (Cell Signaling Technology) was used to determine the effect of INV-403 on the IKK2 activity. Briefly, a biotinylated peptide substrate of IKK2 was phosphorylated by recombinant human IKK2 kinase for 1 hour at 37°C, using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl₂, 3 mM MnCl₂, 3 µM Na-orthovanadate, 1.2 mM DTT, 200 µM ATP, 2.5 µg/50 µl PEG20.000, Substrate: Rb CTF 1.5 µg/50 µl, recombinant IKKbeta: 50 ng/50 µl, INV-403 (variable). The phosphorylated substrate was then Colorimetric ELISA detection methods.

**Quantitative RT-PCR Analysis**
Total RNA was isolated with TRIzol reagent (Invitrogen). Four microgram of total RNA was reverse transcribed by random hexamers and ThermoScript RT-PCR System (Invitrogen). Quantitative real-time PCR was performed with the Stratagene Mx3005 using SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA). Relative expression level compared to GAPDH was obtained as previously described. The primers used in the experiment are depicted in the table below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense/Antisense</th>
<th>Sequence</th>
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<tr>
<td>Rabbit ICAM-1</td>
<td>Sense</td>
<td>5’-GCC TGA GGT CCA GTT CTG TG-3’</td>
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<tr>
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<td>Antisense</td>
<td>5’-GCG GAC ACA GCT CTC AGT AG-3’</td>
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<tr>
<td>Rabbit GAPDH</td>
<td>Sense</td>
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<td></td>
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<td>5’-CCA GCA TCG AAG GTA GAG GA-3’</td>
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<td>5’-TGC CGA GCT AAA TTA CAT ATC G-3’</td>
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<td>5’-TCA TTG TCA CAG AGC CAC CT-3’</td>
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<tr>
<td>Rabbit P-selectin</td>
<td>Sense</td>
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<tr>
<td></td>
<td>Antisense</td>
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<td>Antisense</td>
<td>5’-CCAGTGTTAGGAGTGGC-3’</td>
</tr>
</tbody>
</table>
Supplementary Methods

Reference:


Supplementary Figure I: INV-403 did not change plasma lipoprotein profile in high cholesterol-fed WHHL rabbits.
Supplementary Figure II: INV-403 did not change aortic responses to endothelin (A), SNP (B), PE (C), and insulin (D).