National Cholesterol Awareness Month

Inhibition of Soluble Epoxide Hydrolase Attenuated Atherosclerosis, Abdominal Aortic Aneurysm Formation, and Dyslipidemia

Le-Ning Zhang, Jon Vincelette, Ying Cheng, Upasana Mehra, Dawn Chen, Sampath-Kumar Anandan, Richard Gless, Heather K. Webb, Yi-Xin (Jim) Wang

Objective—Epoxyeicosatrienoic acids (EETs) have been shown to have antiinflammatory effects and therefore may play a role in preventing vascular inflammatory and atherosclerotic diseases. Soluble epoxide hydrolase (s-EH) converts EETs into less bioactive dihydroxyeicosatrienoic acids. Thus, inhibition of s-EH can prevent degradation of EETs and prolong their effects. The present study aimed to test the hypothesis that inhibition of s-EH has vascular protective effects.

Methods and Results—Six-month-old apolipoprotein E–deficient mice were chronically infused with angiotensin II (1.44 mg/kg/d) for 4 weeks to induce abdominal aortic aneurysm (AAA), accelerate atherosclerosis development and carotid artery ligation-induced vascular remodeling. The mice were treated with a novel s-EH inhibitor, AR9276 (1.5 g/L in drinking water) or vehicle for 4 weeks. The results demonstrated that AR9276 significantly reduced the rate of AAA formation and atherosclerotic lesion area, but had no effect on ligation-induced carotid artery remodeling. These effects were associated with a reduction of serum lipid, IL-6, murine IL-8-KC, and IL-1α, and downregulation of gene expressions of ICAM-1, VCAM-1, and IL-6 in the arterial wall.

Conclusions—The present data demonstrate that treatment with an s-EH inhibitor attenuates AAA formation and atherosclerosis development. The attendant downregulation of inflammatory mediators and lipid lowering effects may both contribute to the observed vascular protective effects. (Arterioscler Thromb Vasc Biol. 2009;29:1265-1270.)

Key Words: epoxyeicosatrienoic acids • soluble epoxide hydrolase • dyslipidemia • atherosclerosis • abdominal aorta aneurysm • inflammatory markers

Arachidonic acid can be metabolized by 3 major oxidative pathways: cyclooxygenase (COX) forming prostaglandins; lipoxygenase (LOX) forming hydroxyeicosatetraenoic acids (HETEs) and leukotrienes; and cytochrome P-450 monooxygenase forming epoxides and HETEs. The COX and LOX pathways have been extensively studied, and their eicosanoid products have been shown to play important roles in a variety of biological processes such as inflammation, cell proliferation, and intracellular signaling. In contrast, less is known about the “third pathway” of arachidonic acid metabolism. Recently, epoxyeicosatrienoic acids (EETs), the cytochrome P450 metabolites of arachidonic acid, have received increasing attention for multiple beneficial biological functions, including vasodilation, antiinflammation, and inhibition of proliferation and migration of vascular smooth muscle cells. Based on these properties, it has been postulated that EETs may exert therapeutic benefits in inflammatory vascular diseases, such as atherosclerosis.

Soluble epoxide hydrolase (s-EH) converts EETs into their corresponding dihydroxyeicosatrienoic acids (DHETs), which are generally thought to have reduced biological activity relative to EETs, and hydration of the EETs by s-EH is a dominant mechanism whereby their activity can be reduced. Thus, inhibition of s-EH could be a promising therapeutic target for amplifying the beneficial effects of EETs. Indeed, s-EH inhibitors have been demonstrated to lower blood pressure in hypertension, decrease hypertension-induced renal damage and cerebral ischemia injury, attenuate vascular smooth muscle cell proliferation, and reduce tissue injury associated with lipopolysaccharide-induced systemic inflammation. It has long been recognized that vascular inflammation plays an important role in atherosclerosis and aneurysm formation. However, no studies have been conducted to explore the effects of s-EH inhibitors in these inflammatory vascular diseases. The present study was designed to test the hypothesis that inhibition of s-EH can attenuate the development of atherosclerosis and abdominal aortic aneurysm (AAA) formation in apolipoprotein E (apoE)–deficient mice chronically infused with angiotensin (Ang) II. In addition, the effects of an s-EH inhibitor on carotid artery ligation–induced vascular wall remodeling were examined in the same animal model.
Methods

Experimental Design and Surgical Procedures
All protocols were approved by the institutional animal care and use committee at Arete Therapeutics. Six-month-old male apoE-deficient mice (The Jackson Laboratory, Bar Harbor, Me) fed a normal chow (Harlan Teklad diet #2018, Harlan Laboratories) were used in this study. Baseline blood pressure and body weight were measured before surgery. Animals were anesthetized by inhalation of 2% isoflurane. The left common carotid artery was carefully dissected via a midline neck incision under a dissecting microscope, and then ligated with a 6-0 silk ligature just proximal to its bifurcation. At the time of ligation, a minipump (model 2004, Durect Corp) filled with Ang II (1.44 mg/kg/d) was implanted subcutaneously. The animals were randomly divided into 2 groups: Vehicle, drinking water containing 5% hydroxypropyl-β-cyclodextrin (HPBCD) or AR9276, 1-(1-nicotinoylpiridin-4-yl)-3-(4-trifluoromethoxy)phenylurea, in drinking water containing 1.5 mg/mL. AR9276 in 5% HPBCD. Each experimental group included 11 animals. After 4 weeks of Ang II infusion, systolic blood pressure was measured in conscious mice using a tail-cuff system (Kent Scientific Corporation), and the animals were euthanized. Blood samples were collected via cardiac puncture for the measurement of a serum cholesterol profile (IDEXX Veterinary Services) and serum inflammatory panel (Murigenics) using a mouse cytokine/chemokine panel kit (Millipore), and tissues were removed for analysis (see below).

Quantification of Atherosclerotic Lesions, Ligation-Induced Vessel Wall Remodeling, and Abdominal Aorta Aneurysm Formation

The details are described in supplemental material (available online at http://atvb.ahajournals.org).

Quantitative qRT-PCR
Enzuzhanced mice were perfused transmyocardially with heparinized (20 U/mL) DEPC saline (Biotest Labs). The thoracic aortas were rapidly microdissected from fat and connective tissue and stored in the RNALater (Ambion) for real-time quantitative RT-PCR (Lucy Whittier Molecular & Diagnostic Core Facility/TaqMan Services). The details are described in supplemental material.

Evaluation of the PPARγ Agonist Activity

The PPARγ agonist activity was evaluated using the PolarScreen PPAR Competitor assay, Green (Invitrogen) using the methods as provided by the vendor. AR9276 was tested using a DMSO stock solution, 14, 15 EET was tested using an ethanol stock solution.

Statistics

All results are presented as the mean and standard error (SEM). Comparison between the vehicle and AR9276 treatment groups was performed using unpaired Student’s t test or the Mann–Whitney test for nonparametric data. The percentage of mice that developed AAA was compared between 2 groups using Fisher exact test. Differences were considered statistically significant when the probability value was <0.05.

Results

AR9276 Is a Potent, Selective, and Orally Bioavailable s-EH Inhibitor

The IC50 of AR9276 for inhibition of s-EH was 1.6 nmol/L measured in an enzymatic assay and 8.9 nmol/L in a cell based assay. In off-target screen assays, AR9276 was inactive against over 100 cardiovascular disease related targets, including hydroxy-methylglutaryl-coenzyme A (HMG CoA) reductase at a concentration of 10 μmol/L. In a standard PPARγ activation assay, AR9276 (10 μmol/L) was negative (11%) and 14, 15 EET (1 μmol/L) had a moderate activity (28%) relative to troglitzone, which was used as a standard reference (100%). The pharmacokinetics in mice showed that the clearance and terminal half-life of AR9276 were 0.38 L/h/kg and 4 hours, respectively, after an intravenous administration. The oral bioavailability was more than 100% as calculated by the area under the plasma concentration curve over time (AUC) after an oral dose, relative to the AUC after an intravenous dose. In a separate experiment in apoE-deficient mice, the same dose of AR9276 treatment in drinking water (1.5 g/L, 355 mg/kg daily dose) almost completely abolished whole blood s-EH activity. This was measured as a reduction in the conversion rate of 14, 15 EET to 14, 15 DHET from 28±1.7 nmol/L/min in the vehicle group to 1±0.2 nmol/L/min in the AR9276 group. The average plasma concentration for AR9276 was 21.2±1.8 μg/mL. The chemical name of AR9276 is 1-(1-nicotinoylpiridin-4-yl)-3-(4-trifluoromethoxy)phenylurea, whose detailed physiochemical characterization and chemical structure will be published separately.

| Table. Serum Lipid Panels and Other Parameters in Angiotensin II–Infused ApoE-Deficient Mice Treated With Vehicle or AR9276 |
|-----------------|-----------------|-----------------|
|                  | Vehicle (n=10)  | AR9276 (n=11)   | P Value*   |
| Total cholesterol, mg/dL | 716±82          | 537±27          | 0.04       |
| Triglyceride, mg/dL      | 301±59          | 258±47          | NS         |
| Blood glucose, mg/dL     | 216±22          | 185±17          | NS         |
| Blood pressure, mm Hg    |                 |                 |            |
| Baseline                 | 108±3           | 101±2           | NS         |
| End                       | 137±3           | 130±4           | NS         |
| Body weight, g           | 27±0.7          | 28±0.6          | NS         |
| Daily food consumption, g| 4.2±0.2         | 4.1±0.2         | NS         |

*P value was obtained using Student t test for the statistical comparison between the vehicle and AR9276 groups. NS indicates no statistically significant difference (NS).

AR9276 Lowered Circulating Cholesterol Levels

ApoE-deficient mice spontaneously develop hypercholesterolemia, which is characterized by elevated serum levels of total cholesterol and triglyceride under normal dietary conditions (Table). In mice treated with AR9276 for 4 weeks, the total cholesterol level was significantly lower by 25%, as compared to the vehicle group. There was no significant difference in the triglyceride and glucose levels between the 2 groups. Chronic infusion of Ang II in apoE-deficient mice significantly increased systolic blood pressure. Treatment with AR9276 had no effect on blood pressure, body weight, and food consumption.

AR9276 Attenuated Abdominal Aortic Aneurysm Formation

Chronic infusion of Ang II induced aneurysm formation in the abdominal aorta in 7 of 11 (64%) control apoE-deficient mice. Treatment with AR9276 reduced the incidence of aneurysm formation to 18% (2 of 11). The average outer diameter of the supraprenal aorta was significantly smaller in the AR9276-treated mice than in the vehicle group (Figure 1, top). In mice that developed aneurysm, the severity measured...
by category score was also relatively less in the AR9276-treated mice (majority with type 0 and I) compared to the vehicle group (majority with type III; Figure 1, bottom). There was no type VI aneurysm found in this study.

Histological staining showed that the aortas from the vehicle group had thick walls with intimal plaques, irregular media, and prominent adventitia (Figure 2A). There were foci of acute hemorrhage present in the intima (Figure 2B). The intima was occasionally disrupted by plaques of Mac-3–positive foam cells on the luminal side of the internal elastic lamina (Figure 2C). Prussian blue staining showed iron accumulation (Figure 2D) colocalized with Mac-3–positive staining in the intima and adventitia (Figure 2C). The thickness of the media was increased by extracellular matrix depositing between smooth muscle bundles and stained with trichrome as collagen. Elastin fibers in the media were discontinuous and irregularly oriented. The adventitia was markedly thickened by extracellular matrix that was predominantly collagen. There was a modest increase in adventitial cellularity including fibroblasts and Mac-3–positive mononuclear cells. Segmental regions of the aortic wall showed replacement of the media and adventitia by thick bands of fibroblasts in a collagenous matrix. The aorta from AR9276-treated animals had fewer intimal plaques and no evidence of macrophage and iron accumulation in the intima. These vessels did have medial changes including collagen deposition and some increase in elastin fibers, but the internal elastic lamina generally remained intact and retained a distinctive media of smooth muscle. The adventitia was relatively thin and composed of woven bands of collagen and had less macrophage and no iron accumulation.

AR9276 Reduced Atherosclerotic Lesions in the Aortic Arch and Nonligated Right Carotid Artery

Consistent with previous works,12–14 the nonligated right carotid artery displayed typical and severe fibrous-fatty lesions in the area proximal to the aortic arch and close to the bifurcation. Such atherosclerotic lesions were also observed in the aortic arch. AR9276 treatment significantly reduced atherosclerotic lesion size in both the carotid artery and aortic arch (Figure 3).

AR9276 Had No Effect on Ligation-Induced Vascular Remodeling in the Carotid Artery

Consistent with our previous report,13 ligation of the left carotid arteries for 4 weeks induced vascular remodeling, including neointima formation and adventitial proliferation, leading to expansive remodeling as measured by enlargement of vessel diameter. This was not affected by the AR9276 treatment. The average diameter of the ligated carotid arteries was not significantly different between the 2 test groups (Figure 4).
AR9276 Downregulated the Expression of Proinflammatory Mediators in the Aortic Tissue and in the Blood

Our previous publication reported that chronic infusion with Ang II in apoE-deficient mice significantly upregulated the expression of proinflammatory mediators such as VCAM-1 and ICAM-1 and downregulated the expression of antiinflammatory PPARs.\textsuperscript{14,15} Using the same animal model in the present study, treatment with AR9276 significantly reduced the expression of proinflammatory genes such as VCAM-1, ICAM-1, and IL-6, but did not significantly affect the expression of IL-1\(\alpha\) and PPARs measured in the ascending aortic tissue (Figure 5, top). Consistent with aortic gene expression, circulating protein levels of IL-6 and murine IL-8--KC were also significantly lower in AR9276-treated mice than in the vehicle group (Figure 5, bottom). Interestingly, the serum IL-1\(\alpha\) was reduced in AR9276 group.

Discussion

This study evaluated the effects of a novel s-EH inhibitor, AR9276, in an apoE-deficient mouse model of Ang II--induced aneurysm formation, exacerbated atherosclerosis development and carotid artery ligation--induced vascular remodeling. Our results determined that AR9276 abolished whole blood s-EH activity, attenuated aneurysm formation and atherosclerosis development, but had no effect on ligation-induced vascular remodeling. These effects are associated with a reduction of inflammatory cell infiltration in the vascular wall and downregulation of the expression of proinflammatory mediators in the vasculature and blood, as well as correction of dyslipidemia. These effects were blood pressure--independent because treatment with AR9276 had no significant effect on the pressor effect of Ang II. During preparation of this manuscript, Ulu et al reported that an s-EH inhibitor, AEPU, also reduced the development of atherosclerosis in Ang II--treated apoE-deficient mice, which is consistent with the present results.\textsuperscript{16} However, they found that AEPU did not reduce the plasma cholesterol levels. It could be attributable to the compound specific activity or the animals’ failure to develop hyperlipidemia in this study, which is rare in atherogenic diet--fed apoE-deficient mice.

Vascular inflammation, characterized by an infiltration of monocytes/macrophages and lymphocytes into the arterial wall, is a crucial pathogenic event in atherosclerosis development and AAA formation.\textsuperscript{11,17--19} EETs have been reported to inhibit TNF-\(\alpha\)--induced VCAM-1 and ICAM-1 expression in human endothelial cells.\textsuperscript{20} More recent work has shown that inhibition of s-EH in human coronary endothelial cells markedly increased 14,15-EET levels, which inhibited TNF-\(\alpha\)--mediated generation of the proinflammatory cytokine, IL-8, in human coronary endothelial cells.\textsuperscript{21,22} The present study demonstrated that inhibition of s-EH reduced circulating inflammatory mediators, including IL-1\(\alpha\), IL-6, and murine IL-8--KC, all of which have been reported as being involved in the development of atherosclerosis.\textsuperscript{23--27} Furthermore, the present data have demonstrated that s-EH inhibition decreased the expression of proinflammatory genes in arterial wall, such as VCAM-1 and ICAM-1, which are critical adhesion proteins playing an early and essential role in the pathogenesis of atherosclerosis.\textsuperscript{28} The pathohistological examination also revealed a marked reduction of inflammatory cell infiltration and extracellular matrix destruction in the vascular wall in AR9276-treated mice, which was also accompanied by a reduction of proinflammatory mediators. Taken together, our results strongly suggest that the antiinflammatory action of s-EH inhibition may be one of the key mechanisms mediating the attenuation of atherosclerosis and AAA formation. It has to be pointed that, in the present work, the expression of proinflammatory genes was measured only in the thoracic aortic wall. Although our previous work showed that the expression of proinflammatory genes, such as VCAM-1, was comparable in the aortic arch, thoracic, suprarenal, and infrarenal aorta,\textsuperscript{15} one must be cautious attributing the mechanism(s) underlying the effect of AR9276 on AAA progression based on gene expression in the thoracic aorta. Although vascular inflammation is an important initiating factor in AAA and in the development of atherosclerotic plaques,\textsuperscript{15} matrix metalloproteinases appear to play a more important role in the progression of AAA than in atherosclerosis.\textsuperscript{17,19} AR9276 could be acting on metalloproteinases. In addition, it is difficult to infer mechanism solely on the basis of measurements of gene expression. However, we believe
that the differences in gene expression identified in this study provide reasonable targets for further mechanistic investigation.

Endothelium plays a critical role in the pathophysiology of atherosclerosis and is thus an important target for drugs.29 Our unpublished data demonstrated that treatment of s-EH inhibitors attenuated endothelial dysfunction in several animal models of obese, diabetes, and hypertension. These results indicate that endothelial cells could be one of cell types that mediated the beneficial effects of AR9276. One interesting finding in the present study is iron accumulation in the intima and adventitia of Ang II–induced AAA. Iron accumulation, which has been reported in humans30 and in an Ang II–treated rat model,31 may exacerbate Ang II–induced vascular damage. This can occur through enhancement of oxidative stress by iron-catalyzed hydroxyl radical formation, via Fenton chemistry, and subsequent lipid peroxidation.32 The current work is the first demonstration that Ang II treatment can cause iron accumulation in AAA in an apoE-deficient mouse model and that AR9276 can reduce this iron accumulation.

Hyperlipidemia plays a causal role in progression of atherosclerosis.33 The present data demonstrated that inhibition of s-EH is capable of lowering circulating cholesterol levels, which could also contribute to the attenuation of atherosclerosis and AAA formation. Recent studies suggested that s-EH is involved in cholesterol,34 fatty acid35 and lipid36 metabolism, which may explain the role of AR9276 in lipid control in the present study. Statins prevent the progression of atherosclerosis and coronary artery disease and promote the regression of atherosclerosis presumably via lipid-lowering effects induced by inhibition of HMG CoA reductase.37 However, AR9276 showed no inhibition of HMG CoA reductase activity at a concentration of 10 μmol/L in an off-target screen assay. In contrast to the efficacy of AR9276 in dyslipidemia, simvastatin was found not to reduce cholesterol in apoE-deficient mice.37 Thus, it is unlikely that HMG CoA reductase is a major mediator for the lipid control effects of AR9276 in the present study. However, s-EH has been reported to metabolize the isoprenoid precursors in the cholesterol biosynthesis pathway.33 Thus, the role of AR9276 in cholesterol biosynthesis and metabolic pathway remains to be further investigated.

PPARs have been reported to play an important role in glucose and lipid metabolism as well as in modulation of vascular inflammatory process.38 However, the pleiotropic beneficial vascular effects of PPARs are beyond their actions on lipid and glucose metabolism. Although some s-EH inhibitors have been reported to be capable of activating PPARα, this effect was most likely EET-independent.39 The present data showed that treatment with AR9276 did not significantly affect the expression of PPARs in the aortic wall, indicating that PPARs may not be a major mediator for the antiflammatory, vascular protective and lipid lowering effects of AR9276. Furthermore, both EETs and DHETs have been reported to be capable of activation of PPARα.40,41 Thus, the net contribution to PPAR activation by EETs and DHET after s-EH inhibition remains to be further explored.42

We previously reported that smooth muscle cell migration and proliferation were major pathological processes in this Ang II–accelerated vascular remodeling model.43,44 Davis et al initially reported that EETs and an s-EH inhibitor, 1-Cyclohexyl-3-dodecyl Urea (CDU), attenuated vascular smooth muscle cell proliferation.44 However, they later demonstrated that the antiproliferative effect of CDU is independent of EETs and s-EH.45 This is consistent with our observation that AR9276 had no effect on carotid artery ligation–induced vascular remodeling in Ang II–treated apoE-deficient mice, indicating that s-EH may not be involved in the pathogenesis of vascular smooth muscle cell proliferation in this model.

In summary, the present study demonstrated for the first time that inhibition of s-EH by a novel s-EH inhibitor, AR9276, attenuated atherosclerosis progression and aneurysm formation in apoE-deficient mice chronically treated with Ang II. Inhibition of s-EH by AR9276 inhibited the degradation of EETs, leading to antiinflammatory and lipid lowering effects, both of which may mechanistically contribute to the vascular protective effects of AR9276. Thus, inhibition of s-EH provides a novel therapeutic target for the treatment of inflammatory vascular diseases and dyslipidemia.

Acknowledgments
The authors thank Dr Gabor Rubanyi and Tom Mikita for critically reviewing the manuscript.

Disclosures
The authors are employees of Arête Therapeutics Inc.

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Arterioscler Thromb Vasc Biol. 2009;29:1265-1270; originally published online August 10, 2009;
doi: 10.1161/ATVBAHA.109.186064

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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http://atvb.ahajournals.org/content/29/9/1265

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Quantification of atherosclerotic lesions, ligation-induced vessel wall remodeling, and abdominal aorta aneurysm formation

The right carotid artery, which includes the part of innominate artery and the aortic arch, were dissected, excised, opened longitudinally, and pinned down on wax-coated Petri dishes. Atherosclerotic lesions were visible without staining. Images of the open luminal surface of the vessels were captured with a digital camera (Canon Inc., Japan), mounted on a dissecting microscope. The atherosclerotic lesion area was quantified by use of the Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA) and expressed as a percentage of the total luminal surface area, as described in detail previously 1-3.

The left carotid artery also was dissected, the images were taken with the digital camera and the outer diameter was measured as an index of vessel remodeling.

After the aorta was dissected from the surrounding connective tissue, the images were taken with the digital camera and used later to measure the outer diameter of the suprarenal aorta at the midpoint between the diaphragm and right renal artery. A transverse section of the suprarenal aorta was excised for staining with H&E, elastin-Van Giessen, trichrome, and Prussian Blue as well as immunochemical staining with an anti-macrophage or an anti-actin antibody (Histopathology Reference Laboratory, Hercules, CA). The severity of the aneurysm was assessed with a scoring system as described by Daugherty et al., 4: type 0, no aneurysm (the suprarenal region of the aorta was not obviously different from naïve apoE-KO mice without Ang II treatment); type I, a dilated
lumen with no thrombus; type II, remodeled tissue often containing thrombus; type III, a pronounced bulbous form of type II containing thrombus; and type IV, multiple aneurysms containing thrombus.

Sample collection, automated nucleic acid preparation, and cDNA synthesis

The tissues were added to 500 μl of stabilization solution (1XTransPrep, nucleic acid purification lysis buffer, Applied Biosystems, Foster City, CA) and incubated for 30 minutes at room temperature. Proteinase K and two grinding beads (4 mm diameter, stainless steel beads, SpexCertiprep, Metuchen, NJ) were added, then the tissues were homogenized in a GenoGrinder 2000 (SpexCertiprep, Metuchen, NJ) for 2 min at 1,000 strokes per minute and placed at -20 °C for at least 1 hour to reduce the foam. The protein digest was done at 56 °C for 30 minutes. Total RNA was extracted from 200 μl of diluted (1:2 with 1XTransPrep nucleic acid purification lysis buffer) lysate using a 6100 semi-automated nucleic acid (ANA) workstation (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The following cDNA synthesis protocol was validated through the Lucy Whittier Molecular and diagnostic Core Facility in April of 2008 using a variety of sample types and species. Twelve μL mixed solution (Table one) from each sample was incubated at 42°C for two minutes and briefly centrifuged. A 1μL aliquot was analyzed by TaqMan ® using mouse GAPDH to confirm all gDNA had been digested. Then 8 μL from table two was added to
each well then incubated at 42°C for 40 minutes and inactivated at 95°C for 3 minutes. Finally, 80μL of water was added to each well and mixed thoroughly.

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<th>Table 1</th>
<th>Genomic DNA Elimination Reaction Mix</th>
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<tr>
<td>1x Reagent</td>
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<tr>
<td>1 μL</td>
<td>gDNA WipeOut Buffer</td>
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<tr>
<td>10 μL</td>
<td>Template RNA</td>
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<tr>
<td>1 μL</td>
<td>Rnase free water</td>
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<td>12 μL</td>
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<table>
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<th>Table 2</th>
<th>Reverse Transcription Reaction Mix</th>
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<tr>
<td>1x Reagent</td>
<td></td>
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<tr>
<td>0.5 μL</td>
<td>Quantitect Reverse Transcriptase&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>2 μL</td>
<td>Quantitect RT buffer, 5X</td>
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<tr>
<td>0.5 μL</td>
<td>RT Primer Mix</td>
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<td>0.5 μL</td>
<td>20 pmol Random Primers</td>
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<td>4.5 μL</td>
<td>Rnase free water</td>
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<tr>
<td>8 μL</td>
<td>Total RT mix per sample</td>
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<sup>a</sup>- Enzyme added prior to use.

**Real-time TaqMan ® PCR**

Pre-validated gene expression assays were purchased from Applied Biosystems for IL-1α (Mm00439620_m1) and GAPDH (Mm99999915_g1). The Real Time TaqMan ® PCR assays used for mouse ICAM-1 (NM_010493.2), IL-6 (NM_031168), PPAR-α (NM_011144), PPAR-γ (NM_011146) and VCAM-1 (NM011693.3) were designed using AB Primer Express 2 by the Lucy Whittier Molecular and Diagnostic Core Facility with the sequences from the listed genebank accession numbers. As an additional precautionary step, the probe of each assay spans an exon-exon junction to restrict the TaqMan PCR specificity.
to cDNA. In addition, an aliquot of the cDNA from a subset of samples was TaqMan ® analyzed with mouse GAPDH.

Each PCR reaction contained 20X primers and probes for the respective TaqMan ® system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan ® probe and commercially available PCR mastermix (TaqMan ® Universal PCR Mastermix, Applied Biosystems, Foster City, CA) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 μl of the diluted cDNA sample in a final volume of 12 μl. The samples were placed in a 384 well plate and amplified in an automated fluorometer (ABI PRISM 7900 HTA FAST, ABI, Foster City, CA). ABI’s standard amplification conditions were used: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Fluorescent signals were collected during the annealing temperature and CT values exported with a threshold of 0.2 for IL-1α, 0.1 for ICAM-1 & PPAR-γ, 0.06 for VCAM-1, 0.04 for PPAR-α & IL-6 and a baseline value of 3-15. For GAPDH, a threshold of 0.1 and baseline value of 3-10 was used.

**Relative quantitation of gene transcription**

Final quantitation was done using the comparative CT method (User Bulletin #2, Applied Biosystems, Foster City, CA), and is reported as relative transcription or the n-fold difference relative to a calibrator cDNA. Prior to profiling, a
housekeeping validation experiment was run using three samples from each
group which were then TaqMan analyzed with Act b, B2M, GAPDH, and HPRT1.
The most stable housekeeping gene, GAPDH (with a standard deviation of 0.38
and an average of 18.98 from three samples from each group), was used to
normalize the Ct values of the target genes (ΔCT). The ΔCT was calibrated
against the average of the control group within each target gene. The relative
linear amount of target molecules relative to the calibrator, was calculated by $2^{-\Delta\Delta CT}$.
Therefore, all gene transcription is expressed as an n-fold difference
relative to the calibrator. Linearized values greater than one indicate increased
expression and linearized values less than one indicate decreased expression.

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