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Objective—Inflammatory processes and foam cell formation are key determinants in the initiation and progression of atherosclerosis. Electrophilic nitro–fatty acids, byproducts of nitric oxide- and nitrite-dependent redox reactions of unsaturated fatty acids, exhibit antiinflammatory signaling actions in inflammatory and vascular cell model systems. The in vivo action of nitro–fatty acids in chronic inflammatory processes such as atherosclerosis remains to be elucidated.

Methods and Results—Herein, we demonstrate that subcutaneously administered 9- and 10-nitro-octadecenoic acid (nitro-oleic acid) potently reduced atherosclerotic lesion formation in apolipoprotein E–deficient mice. Nitro–fatty acids did not modulate serum lipoprotein profiles. Immunostaining and gene expression analyses revealed that nitro-oleic acid attenuated lesion formation by suppressing tissue oxidant generation, inhibiting adhesion molecule expression, and decreasing vessel wall infiltration of inflammatory cells. In addition, nitro-oleic acid reduced foam cell formation by attenuating oxidized low-density lipoprotein–induced phosphorylation of signal transducer and activator of transcription-1, a transcription factor linked to foam cell formation in atherosclerotic plaques. Atherosclerotic lesions of nitro-oleic acid-treated animals also showed an increased content of collagen and α-smooth muscle actin, suggesting conferral of higher plaque stability.

Conclusion—These results reveal the antiatherogenic actions of electrophilic nitro–fatty acids in a murine model of atherosclerosis. (*Arterioscler Thromb Vasc Biol*. 2010;30:00-00.)

Key Words: nitro–fatty acids ■ atherosclerosis ■ foam cells ■ inflammation ■ oxidative stress ■ electrophile

N itro-fatty acid derivatives (NO_2-FA) are electrophilic lipid signaling mediators generated endogenously by nitric oxide (NO) and nitrite (NO_2^-)-derived reactive species during oxidative stress.[1] NO_2-FA signal via predominantly antiinflammatory mechanisms that inhibit neutrophil activation, platelet aggregation, and macrophage activation.[2–5] NO_2-FA also activate peroxisome proliferator–activated receptor-γ and induce heme oxygenase-1 expression, both critical mediators of antiinflammatory actions in the vasculature.[6–8]

NO_2-FA mainly exert cell signaling actions by a covalent and reversible posttranslational modification (S-alkylation) of key signaling proteins, because of a kinetically rapid electrophilic reactivity.[9,10] Thiol residues of proteins are highly susceptible nucleophilic targets of electrophile reaction, with S-alkylation frequently altering protein structure and function. For example, (1) NO_2-FA covalently bind the p65 subunit of nuclear factor κB, inhibiting DNA binding activity, repressing nuclear factor κB–dependent gene expression, and suppressing downstream proinflammatory reactions such as macrophage cytokine and nitric oxide synthase-2 expression;[3] (2) NO_2-FA S-alkylate critical thiols of Keap-1, inducing the release of Nrf2 and activation of antioxidant response element–dependent gene products, a reaction that in turn mitigates inflammatory responses;[11] (3) NO_2-FA act as agonists of peroxisome proliferator–activated receptor-γ by S-alkylating peroxisome proliferator–activated receptor-γ at Cys285;[12] and (4) NO_2-FA serve as robust small molecule inducers of heat shock response gene expression.[13] Finally, NO_2-FA inhibit the phosphorylation of the proinflammatory signal transducer and activator of transcription-1 (STAT-1) in lipopolysaccharide (LPS)-stimulated macrophages.[14] Notably, STAT-1 activation is closely linked to foam cell formation in murine models of atherosclerosis.[15–17]

Atherosclerosis is a chronic inflammatory disease, in which leukocyte activation plays an important role in athero-

sclerotic lesion initiation and progression.[18] Because NO_2-FA inhibit neutrophil function and monocyte adhesion to endothelial cells in vitro, by suppressing adhesion molecule expression and cytokine release from activated monocytes/macrophages,[3] we investigated the effect of nitro-oleic acid (OA-NO_2) on atherosclerotic lesion formation in apolipoprotein E–deficient (apoE$^{-/-}$) mice.

Herein we reveal that OA-NO_2 significantly reduced atherosclerotic lesion formation by attenuating the accumulation of inflammatory cells within atherosclerotic lesions and limiting the expression of adhesion molecules and monocyte chemoattractant protein-1 (MCP-1). Moreover, OA-NO_2 inhibited oxidized low-density lipoprotein (oxLDL)–induced phosphorylation of STAT-1 both in vitro and in vivo, a reaction that minimizes lipid accumulation in macrophages and inhibits foam cell formation. These results support the idea that electrophilic lipid oxidation and nitration products stemming from oxidative inflammatory reactions can serve to induce adaptive and antiinflammatory actions in cardiovascular diseases.

Methods

Animals and Experimental Design
Male apoE$^{-/-}$ mice (8 weeks of age, Jackson Laboratory) fed an atherogenic diet for 12 weeks (21% fat and 1.25% cholesterol, Harlan Teklan) were treated with OA-NO_2 (8 mg/kg/d), OA (8 mg/kg/d, Nu Chek Inc), or vehicle (polyethylene glycol/ethanol) via subcutaneously implanted osmotic mini-pumps (Alzet, model 2004). OA-NO_2 was synthesized via nitroselenation, giving an equimolar distribution of 9- and 10-nitro-octadecenoic acid regioisomers.[8] All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (approval 0709432).

Lesion Analysis
Atherosclerotic lesions at the level of the aortic valve were detected by Oil Red O staining. For lesion quantification, 4 sections cut every 20 μm from 1 animal were analyzed (n=14 animals per treatment group). For determination of atherosclerotic plaque area in the entire aorta, aortas were dissected and stained en face with Sudan (n=6 per group). Quantitative analysis of plaque area was performed by 2 blinded observers using image analysis software (MetaMorph software, MDS).

Histology, Immunofluorescence, and Western Blot Analysis
Aortic root sections were stained with Trichrome, DHE (Sigma), and specific antibodies against monocytes/macrophages (MOMA-2, Serotec), vascular cell adhesion molecule (VCAM) (Santa Cruz Biotechnology), intercellular adhesion molecule (ICAM) (Abcam), neutrophils (Ly6G; Abcam), α-smooth muscle actin (α-SMA; Sigma), 3-nitrotyrosine, and pSTAT-1 (Tyr701-phosphorylation; Cell Signaling Technology). Addition of polyethylene glycol–superoxide dismutase served as control for nonspecific superoxide detection by DHE oxidation. Color threshold and planimetry were used for quantification. All quantifications were performed by using MetaMorph software (MDS), because this program is able to automatically detect positively stained areas/cells by setting specific color thresholds. For Western blot analysis, a specific antibody against VASP (Immunoglobe) was used.

Metabolic Measurements
Plasma triacylglyceride and cholesterol levels were determined after a 6-hour fast using enzyme-based assays (Triglyceride Assay Kit, Cayman; EnzymChrom Cholesterol Assay Kit, BioAssay Systems).

Blood glucose was assessed by a TrueTrack glucometer (Home Diagnostics Inc). Fast protein liquid chromatography analysis was performed to assess lipoprotein profiles.

Quantitative Real-Time PCR
Aortas (n=10 per group) were dissected, and RNA was extracted by TRIzol (Invitrogen) and reverse transcribed using the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. Real-time PCR was performed with TaqMan Fast Universal PCR Master Mix using gene expression assays for ICAM, VCAM, CD68, and MCP-1 normalized to β2-microglobulin. Samples were run in duplicate on the StepOne detection system (Applied Biosystems).

Assessment of Lipid Accumulation in Isolated Peritoneal Macrophages
Three days after intraperitoneal thioglycollate injection, peritoneal macrophages were harvested from OA-NO_2- or vehicle-treated apoE$^{-/-}$ mice fed a high-fat diet (4 weeks of treatment, n=4 per group). Following collection, peritoneal macrophages were immediately seeded at the same density into 6-well plates with 10% FBS/DMEM, and after attachment, cells were fixed and stained for lipids using Oil Red O staining.

In Vitro Experiments
Bone marrow-derived macrophages (BMDMs) were isolated from apoE$^{-/-}$ mice following 4 weeks of treatment with OA-NO_2 or vehicle. BMDMs were grown in 20% FBS/DMEM media (Cellgro) containing special growth factors derived from cultured CCL-1 cells (American Type Culture Collection). BMDMs were cultured no longer than 25 days before use. For Western blot analysis of STAT-1 and phosphorylated STAT-1 (anti-STAT-1 antibody, Tyr701, anti-pSTAT-1 antibody; Cell Signaling Technology), BMDMs were treated with vehicle, oxLDL (100 μg/mL), OA-NO_2 (500 nmol/L, 1000 nmol/L), or a combination of oxLDL and OA-NO_2 for 2 hours in 10% FBS/DMEM. LPS (100 ng/mL) served as positive control. Densitometric analysis using ImageJ software (National Institutes of Health) was applied to assess differences between the treatment groups.

Native low-density lipoprotein (LDL) was collected with University of Pittsburgh institutional review board approval (PRO07110032) from healthy volunteers following a modified protocol, as described previously.[19] In brief, after addition of diethylene triamine pentaacetic acid, plasma density was adjusted to 1.21 g/mL using potassium bromide. After ultracentrifugation, the LDL fraction was collected, concentrated, and dialyzed. Purified LDL was filtered, and protein concentration was determined. Following oxidation by cupric chloride, agarose gel electrophoresis revealed a relative electrophoretic mobility shift of 1.5 for oxLDL compared with native LDL.

Superoxide production by BMDMs was determined via spectrophotometric analysis of cytochrome c reduction[20] following 6 hours of incubation with LPS (20 ng/mL) alone or in combination with OA-NO_2 (25 and 50 nmol/L) in serum-free medium.

Statistical Analysis
Data are presented as mean$\pm$SEM. Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test. Statistical analysis was performed using 1-way ANOVA followed by the Bonferroni post hoc test or unpaired Student t test, as appropriate. A value of $P<0.05$ was considered statistically significant. All calculations were carried out using SPSS version 15.0.

Results

OA-NO_2 Decreases Atherosclerotic Lesion Formation
Male apoE$^{-/-}$ mice fed an atherogenic high-fat diet were treated with either vehicle, OA or OA-NO_2 (n=14 animals
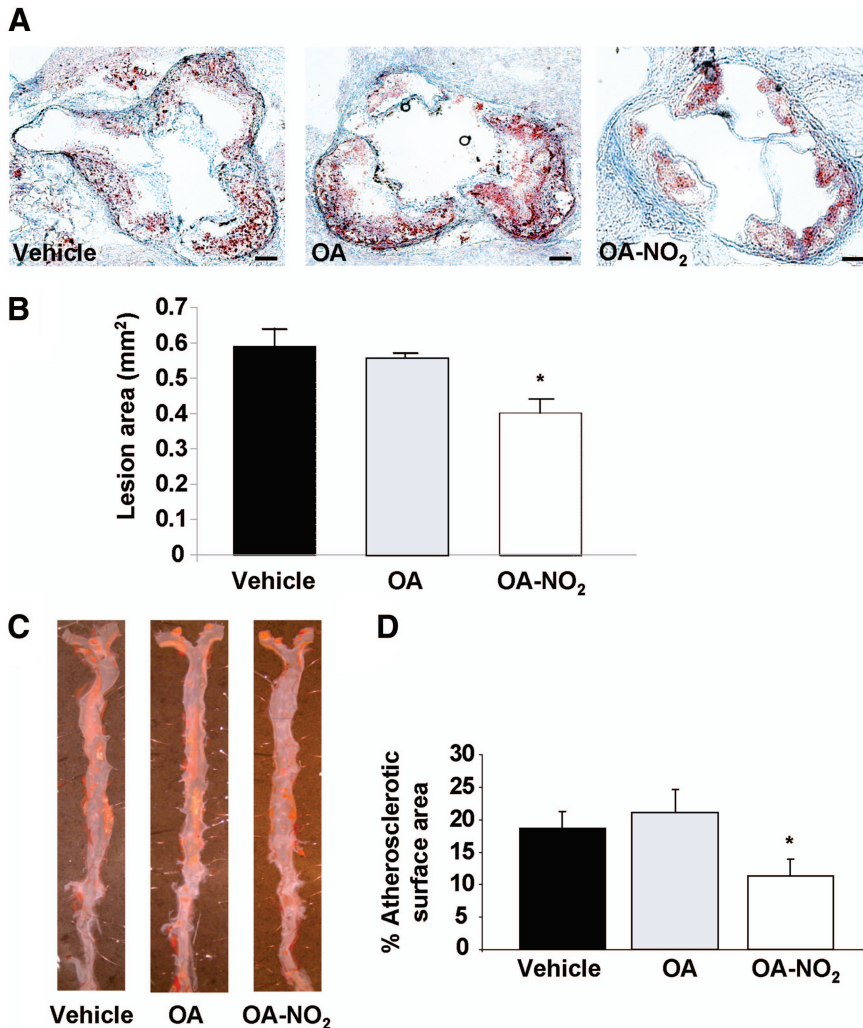
Figure 1. OA-NO$_2$ inhibits atherosclerotic lesion formation. A, Representative Oil Red O–stained aortic sections (magnification ×10; scale bar indicates 100 μm). B, In apoE$^{-/-}$ mice (n=14 per group) fed an atherogenic diet for 12 weeks, OA-NO$_2$ significantly reduced aortic lesion area compared with OA and vehicle ($P<0.001$; *$P=0.003$ versus vehicle; $P=0.015$ versus OA). C, Representative en face Sudan staining of the whole aorta. D, Quantification of atherosclerotic lesions revealing significantly less atherosclerotic area in OA-NO$_2$-treated mice ($P<0.001$; *$P<0.001$ versus vehicle and OA; n=6 per group).

per group) for 12 weeks. OA-NO$_2$ significantly inhibited the formation of atherosclerotic lesions in the aortic root by 32% compared with vehicle and OA (lesion area OA-NO$_2$: 0.40±0.04 mm^2; vehicle: 0.59±0.05 mm^2; OA: 0.56± 0.01 mm^2; $P=0.002$; Figure 1A and 1B). There were no significant differences between vehicle- and OA-treated animal lesion areas. En face Sudan staining of whole aortas (n=6) also revealed a significant 39% reduction of atherosclerotic plaque formation in OA-NO$_2$-treated animals ($P<0.001$; Figure 1C and 1D).

OA-NO$_2$ Does Not Influence Metabolic Profile

To test whether OA-NO$_2$–induced reduction of atherosclerotic lesion formation was due to metabolic effects, fasting plasma lipid and glucose levels were determined. Total cholesterol and total triacylglyceride levels after 12 weeks of treatment were not affected by OA-NO$_2$ treatment. Of note, OA significantly increased plasma triacylglyceride levels after 12 weeks, compared with vehicle and OA-NO$_2$ ($P<0.033$; Figure 2A and 2B). Fast protein liquid chromatography analysis showed no impact of OA-NO$_2$ or control treatments on the plasma lipoprotein profile (Figure 2C).

There were also no differences in blood glucose levels (baseline: OA-NO$_2$, 116.3±4.6 mg/dL; vehicle, 113.6±7.4 mg/dL; OA, 115.7±6.0 mg/dL; $P=0.95$; 12 weeks: OA-NO$_2$, 106.9±5.3 mg/dL; vehicle, 111.9±5.1 mg/dL; OA, 107.7±5.8 mg/dL; $P=0.78$), food consumption, or weight gain among the 3 groups (weight, baseline: OA-NO$_2$, 22.4±0.4 g; vehicle, 22.2±0.6 g; OA, 22.1±0.4 g; $P=0.93$; 12 weeks: OA-NO$_2$, 30.7±0.3 g; vehicle, 30.9±0.7 g; OA, 30.3±0.4 g; $P=0.68$).

Antiinflammatory and Antioxidant Actions of OA-NO$_2$ Inhibit Atherosclerotic Lesion Formation

Inflammatory processes within the vessel wall mediate the initiation and progression of atherosclerosis. In this context, monocyte invasion into lesion-prone areas in the arterial wall and differentiation of monocytes into resident macrophages contributes to atherosclerotic plaque development.[18,21] The vessel wall adhesion of monocytes is mediated primarily by VCAM-1.[22,23] To elucidate mechanisms contributing to OA-NO$_2$-induced decreases in atherosclerotic lesion formation, the influence of OA-NO$_2$ on monocyte recruitment to atherosclerotic lesions was investigated. In particular, the effect of OA-NO$_2$ on monocyte/ macrophage (MOMA) accumulation in atherosclerotic plaques and the expression of VCAM-1 and MCP-1 in the aortic wall was determined. Quantitative immunofluores-
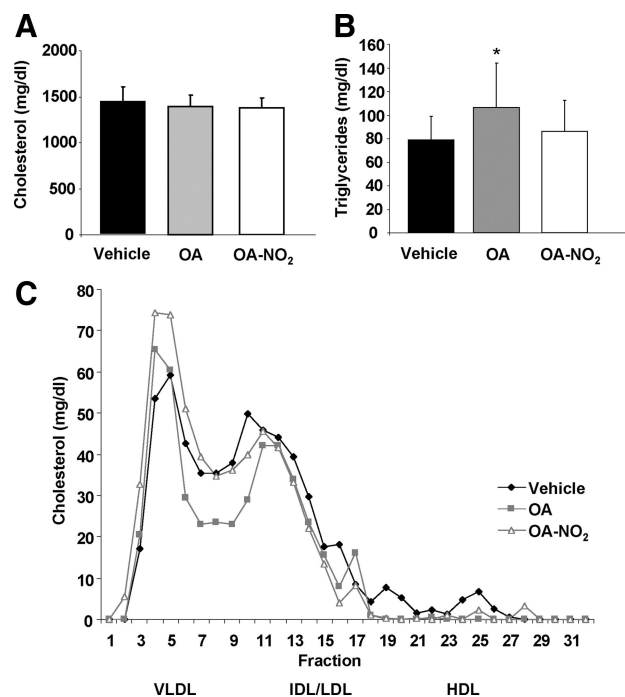
Figure 2. Metabolic profile was not influenced by OA-NO$_2$. A and B, Levels of total plasma cholesterol and triacylglycerides were not changed by OA-NO$_2$ ($P=0.03$; *$P=0.035$ versus vehicle). C, Fast protein liquid chromatography analysis showed no impact of any treatment on the cholesterol profile. VLDL indicates very low-density lipoprotein.

Figure 3. Monocyte and macrophage accumulation in atherosclerotic plaques was reduced by OA-NO$_2$. A, Representative immunostained aortic sections (magnification ×10 and ×20; scale bar indicates 100 μm; blue=nuclei, green=MOMA). B, Quantification of MOMA-positive area relative to lesion area. OA-NO$_2$ significantly reduced MOMA-positive area ($P<0.001$; *$P<0.001$ versus vehicle and versus OA). C, Real-time PCR showed a significant reduction in CD68 and MCP-1 expression in the aortic wall ($P<0.05$; *$P<0.05$ versus vehicle and versus OA).

cent imaging revealed that the area of monocytes/macrophages within the lesion area was significantly decreased in mice treated with OA-NO$_2$ compared with the OA and vehicle treatment groups ($P<0.001$; Figure 3A and 3B). This was confirmed by quantitative real-time PCR analysis showing significantly lower expression of CD68 mRNA in the aortas of OA-NO$_2$-treated animals ($P=0.03$; Figure 3C). OA-NO$_2$ also significantly decreased levels of MCP-1 mRNA ($P<0.05$; Figure 3C). In accordance with previous in vitro observations,[3] OA-NO$_2$-treated animals also displayed decreased VCAM-1 expression in the aortic wall, as assessed by immunostaining and quantitative real-time PCR (Figure 4A and 4B).

The invasion of neutrophils into the arterial wall is facilitated by ICAM-assisted trafficking, an event that also contributes to atherosclerotic plaque initiation and progression.[18,24] Immunofluorescent analysis and quantitative real-time PCR showed decreased ICAM expression in the aortic wall (Figure 4B and 4C) in concert with a reduction in neutrophil accumulation in atherosclerotic lesions of OA-NO$_2$-treated animals (Figure 4C and 4D). In the aggregate, these findings reinforce the precept that the antiinflammatory signaling actions of OA-NO$_2$ mediate a reduction in atherosclerotic lesion formation and development.

DHE and 3-nitrotyrosine staining were used to reflect the extents of generation of superoxide and both peroxynitrite- and heme peroxidase–catalyzed protein nitration reactions occurring in aortic root sections (Figure 4E). These indices revealed that OA-NO$_2$-dependent signaling actions ultimately led to the att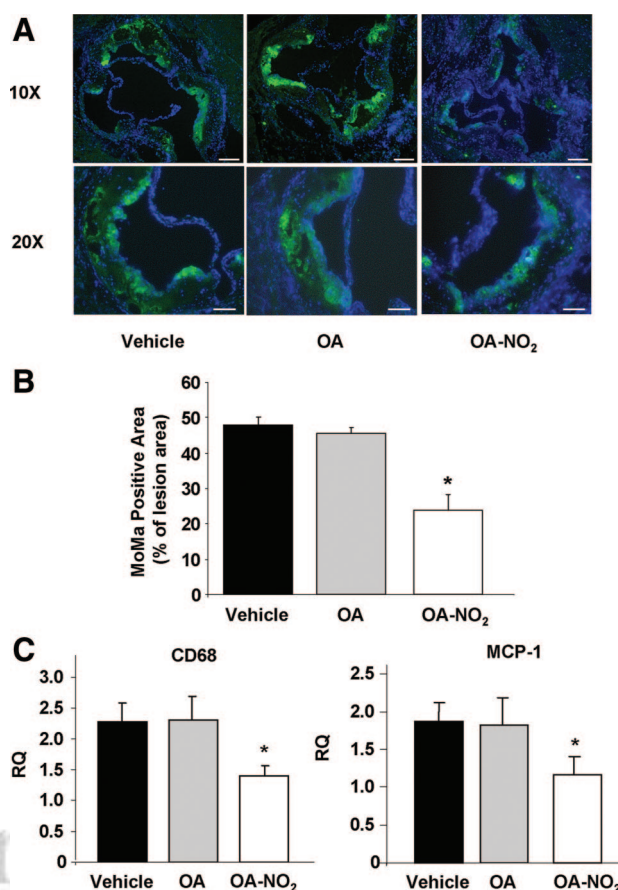enuation of the generation of reactive oxygen species and downstream nitration reactions, events that may also contribute to the net antiatherosclerotic actions of nitro–fatty acid derivatives. This OA-NO$_2$-mediated response, observed in vivo, was also reflected by supporting in vitro studies. LPS-stimulated BMDMs, isolated from apoE$^{-/-}$ mice and treated with OA-NO$_2$, displayed attenuated rates of superoxide generation (Figure 4F; $P<0.004$). Furthermore, the assessment of the expression of VASP and phosphorylated VASP in aortic tissue indicated a trend toward greater extents of NO bioavailability in mice treated with OA-NO$_2$ ($P=0.08$); however, endothelial function was not assessed directly in this study.

In addition to inhibiting plaque formation, OA-NO$_2$ also increased plaque stability, a clinically-significant event that is attributed in part to extents of collagen synthesis by smooth muscle cells.[25] Trichrome staining revealed that the collagen content of atherosclerotic lesions in animals treated with OA-NO$_2$ was significantly greater. OA-NO$_2$ treatment also resulted in an increased content of α-SMA within individual plaques (Figure 5A and 5B).
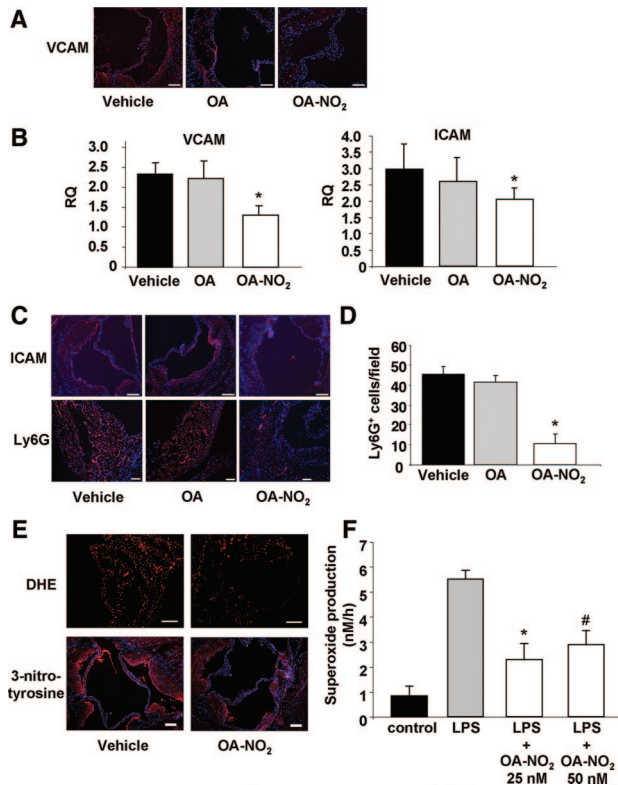
Figure 4. VCAM and ICAM were downregulated by OA-NO$_2$. A, Representative immunostained aortic sections using specific antibodies against VCAM (magnification ×20; scale bar indicates 100 μm; blue=nuclei, red=VCAM). B, VCAM and ICAM mRNA expression in the aortic wall was lower in OA-NO$_2$-treated animals ($P<0.05$; *$P<0.05$ versus vehicle and versus OA). C, Representative immunostained aortic sections using ICAM and Ly6G antibodies showed less ICAM expression and neutrophil infiltration in OA-NO$_2$-treated animals (magnification ×20; scale bar indicates 100 μm; blue=nuclei, red=ICAM/neutrophils). D, Densitometric quantification of Ly6G positive cells per field of view ($P<0.001$; *$P<0.001$ versus vehicle and versus OA). E, Representative immunostaining for DHE (magnification ×20; scale bar indicates 100 μm) and 3-nitrotyrosine (magnification ×10; scale bar indicates 100 μm; blue=nuclei, red=3-nitrotyrosine). F, Ex vivo LPS-induced superoxide production by BMDMs isolated from apoE$^{-/-}$ mice with and without OA-NO$_2$ treatment ($P=0.004$; *$P=0.003$ versus LPS; #$P=0.07$ versus LPS).

Foam Cell Formation Is Inhibited by OA-NO$_2$ In Vitro and In Vivo

The transformation of invading macrophages into foam cells by oxLDL uptake and the subsequent activation-induced expression and secretion of multiple inflammatory mediators is a key aspect of atherogenesis. To determine whether OA-NO$_2$ influences foam cell formation and function, beyond inhibition of monocyte/macrophage infiltration, peritoneal macrophages and BMDMs isolated from apoE$^{-/-}$ mice were studied. Peritoneal macrophages from OA-NO$_2$-treated mice showed markedly reduced intracellular lipid accumulation compared with controls. (Figure 6A). To further elucidate the mechanism of reduced foam cell formation, the impact of OA-NO$_2$ on oxLDL-induced phosphorylation of STAT-1 was determined. Western blot analysis, confirmed by densitometry, showed that OA-NO$_2$ decreased phosphorylation of STAT-1 in BMDMs (Figure 6B; $P=0.001$). Im-

munofluorescent analysis also revealed that phosphorylation of STAT-1 was reduced in atherosclerotic plaques of OA-NO$_2$-treated mice (Figure 6C).

Discussion

The extended exposure of apoE$^{-/-}$ mice to low concentrations of an electrophilic nitro–fatty acid, OA-NO$_2$, markedly reduced atherosclerotic lesion formation and enhanced expression of proteins associated with plaque stability. The antiatherosclerotic actions of OA-NO$_2$ were linked with decreased adhesion molecule expression, vessel wall deposition of inflammatory cells and oxidative stress. Finally, OA-NO$_2$ treatment further diminished foam cell formation by inhibition of oxLDL-induced STAT-1 phosphorylation.

Atherosclerosis has long been associated with excess vascular production of reactive oxygen species and oxides of nitrogen.[26,27] Secondary nitric oxide- and nitrite-derived nitrating species stemming from this inflammatory milieu nitrates not only protein tyrosine residues, but also unsaturated fatty acids, yielding electrophilic products that react rapidly and reversibly with thiols of critical signaling mediators.[9,28] Inflammatory conditions that have been modeled in vitro and studied in vivo reveal increased nitration of fatty acids.[1] Acceleration of nitration reactions can also be promoted by the hydrophobic milieu of cell membranes and lipoproteins because of the greater local concentrations of reactants, such as oxygen and nitric oxide.[29,30] Because the second-order rate constants for thiol reaction of the endogenously formed nitro–fatty acid derivatives oleic acid and linoleic acid are similar,[9] OA-NO$_2$ was selected for extended administration in vivo via osmotic minipump because of the greater stability and resistance of the monounsaturated oleic acid to secondary oxidation reactions. Because of this kinetically rapid and reversible electrophilic reaction with thiols, NO$_2$-FA readily modify biological targets posttranslationally.[9,10,28] To date, multiple electrophile-sensitive transcriptional regulatory factors that mediate defense against or resolution of inflammation have been identified as targets of NO$_2$-FA.[3,11] Several transcription factors that regulate adaptive and antiinflammatory signaling reactions possess functionally significant electrophile-reactive amino acids that are critical for DNA binding and gene expression. These highly conserved signaling mediators evolved to facilitate tissue responses to oxidizing and electrophilic species generated by inflammatory and metabolic stress.[31]

Although atherosclerosis was formerly regarded as a lipid accumulating disease, it is now widely accepted that inflammatory processes play a critical role in all phases of atherosclerosis. After cell adhesion molecule–mediated recruitment and vessel wall infiltration, leukocytes propagate the formation of inflammatory stimuli that in turn instigate a cascade of reactions ultimately responsible for the development of atherosclerotic plaques.[15,32,33] In the present study, OA-NO$_2$ potently inhibited the expression of cell adhesion molecules. There was also reduced monocyte/macrophage and neutrophil accumulation in the atherosclerotic lesions of animals treated with OA-NO$_2$. These findings are concordant with
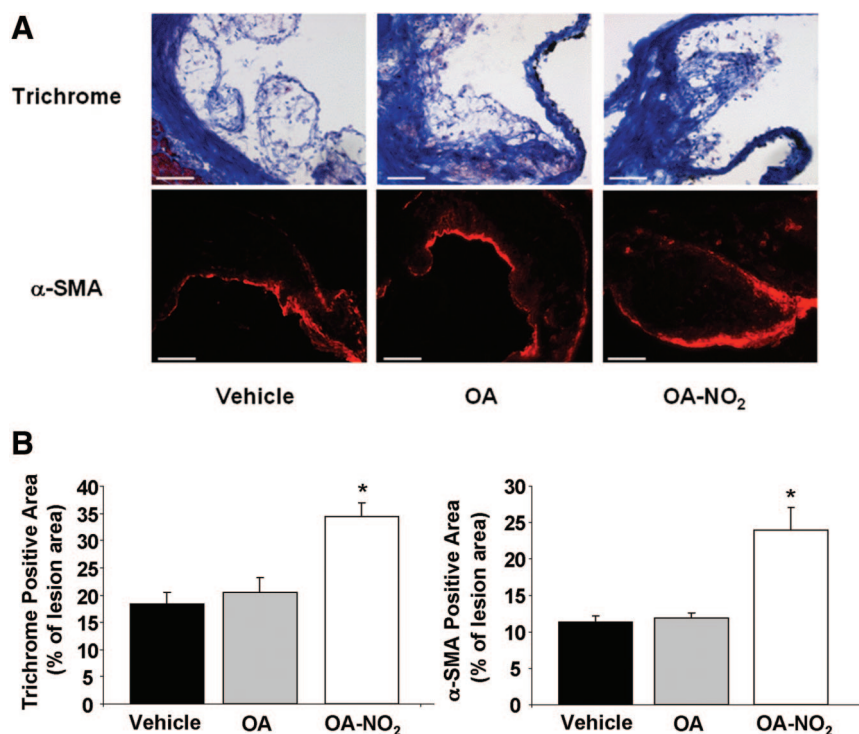
A

B

Figure 5. OA-NO$_2$ increased plaque stability. A, Representative aortic sections stained by Trichrome and a α-SMA-specific antibody (magnification $\times$20; scale bar indicates 100 μm). B, Quantification revealed a significantly larger collagen and α-SMA positive area in animals treated with OA-NO$_2$ ($P<0.001$; *$P<0.001$ versus vehicle and versus OA).

previous in vitro results, where NO$_2$-FA decreased endothelial VCAM expression and inhibited monocyte adhesion to endothelial cell monolayers.[3] The current data also reinforce the concept that NO$_2$-FA inhibit monocyte/macrophage activation, because there was a significant reduction in MCP-1 expression in OA-NO$_2$-treated animals. In addition to these antiinflammatory properties, OA-NO$_2$ displayed indirect antioxidant effects in vivo, also potentially contributing to the observed antiatherosclerotic actions of OA-NO$_2$. Current data do not indicate direct free radical or oxidant scavenging properties of nitro–fatty acids, beyond the initial and quantitatively small consumption of nitrogen dioxide ($\cdot$NO$_2$) that would occur during unsaturated fatty acid nitration.[1] Once formed, nitroalkene derivatives of fatty acids convey indirect but potentially highly potent antioxidant effects via their signaling actions as electrophiles.[34] Cell and tissue responses that have been defined for nitro–fatty acids could result in apparent antioxidant effects, including receptor-dependent (peroxisome proliferator–activated receptor-γ), transcription factor-mediated (nuclear factor κB, Nrf2, heat shock factor), and enzyme catalytic (xanthine oxidoreductase) modulatory actions of these electrophilic species.[1,13,34,35]

In addition to a marked reduction in atherosclerotic lesion formation, the present data also suggest that NO$_2$-FA can have an impact on plaque stability, because there was an increased collagen and α-SMA content—surrogates for plaque stability. The inhibition of collagen synthesis and/or increased catabolism of extracellular matrix by metalloproteinases are factors that lead to plaque instability and can result in plaque rupture and consequent acute occlusion of the vessel.[27,36–39] Our data reveal that these processes are inhibited by NO$_2$-FA administration.

OA-NO$_2$ also further influenced foam cell formation, beyond inhibiting inflammatory cell invasion and activation.

During atherogenesis, macrophages ingest and accumulate oxLDL by different receptor-dependent mechanisms, ultimately transforming into lipid-rich foam cells, a process under stringent regulation by STAT-1-mediated signaling reactions.[17] Of note, the Janus kinase–STAT pathway has also been identified as a critical mediator of interferon-γ-dependent signaling, which propagates initiation and progression of atherosclerotic plaque formation. This in turn induces the further release of proatherogenic chemokines such as MCP-1 and stimulates the apoptosis of plaque-associated macrophages, in turn enhancing the progression of lesion formation.[16] Previous in vitro studies revealed that nitro–fatty acids, and not native fatty acid controls, potently inhibited LPS-induced STAT-1 phosphorylation. For this reason, the effect of OA-NO$_2$ on oxLDL-induced STAT-1 phosphorylation was evaluated.[14] Both in vivo and in vitro-based studies revealed that peritoneal macrophages isolated from OA-NO$_2$-treated animals displayed less intracellular lipid accumulation and that the oxLDL-induced phosphorylation of STAT-1 was suppressed by OA-NO$_2$. These findings indicate that OA-NO$_2$ can in part limit foam cell formation by interfering with STAT-1 activation, with this effect ultimately being translated into a marked reduction in atherosclerotic lesion formation.

Our study of serum lipid profiles and blood glucose levels did not provide any evidence that NO$_2$-FA influenced the metabolic profile of apoE$^{-/-}$ mice, affirming the concept that the protective antiatherosclerotic actions of NO$_2$-FA were due to their pluripotent antiinflammatory signaling properties.

In conclusion, the present study reveals that in vivo administration of nanomolar levels of electrophilic NO$_2$-FA potently inhibits atherosclerotic lesion formation and induces plaque stability in a murine model of atherosclerosis by targeting several pathways involved in atherogenesis. Be-
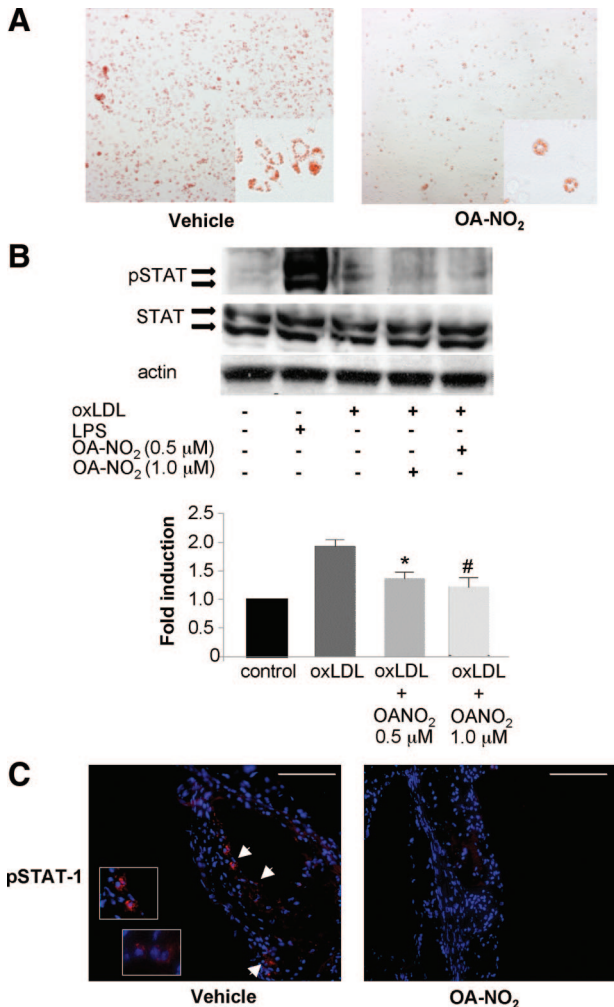
Figure 6. OA-NO$_2$ reduced foam cell formation in vivo and inhibited phosphorylation of STAT-1 in vitro as well as in vivo. A, Representative Oil Red O–stained peritoneal macrophages harvested from apoE$^{-/-}$ mice after 4 weeks of treatment. OA-NO$_2$ reduced intracellular lipid accumulation. B, OA-NO$_2$ decreased oxLDL-induced phosphorylation of STAT-1 in BMDMs. One representative blot is shown. Densitometric analysis of 3 independent experiments ($P=0.001$; *$P=0.02$; #$P=0.006$ versus oxLDL). C, Phosphorylation of STAT-1 was also reduced in vivo as shown by immunofluorescence. Arrows indicate pSTAT-1 positive cells (magnification $\times20$ and $\times60$; scale bar indicates 100 μm; blue=nuclei, red=pSTAT-1).

cause antiatherogenic and cardioprotective dietary omega-6 and omega-3 polyunsaturated fatty acids[40] can also be endogenously oxidized and nitrated to electrophilic derivatives such as α,β-unsaturated ketone and NO$_2$-containing derivatives, it is intriguing to speculate that these redox-derived byproducts and their downstream signaling actions contribute to the salutary cardiovascular actions of polyunsaturated fatty acids. Because human atherosclerosis is primarily a consequence of vessel wall inflammation, NO$_2$-FA administration may thus represent a useful therapeutic strategy for avoiding or treating atherosclerosis.

Acknowledgments

Disclosures
B.A.F. has a financial interest in Complexa, Inc.

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