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Nuclear Factor- κ B Activation Contributes to Vascular Endothelial Dysfunction via Oxidative Stress in Overweight/Obese Middle-Aged and Older Humans

Gary L. Pierce, PhD; Lisa A. Lesniewski, PhD; Brooke R. Lawson, MS;
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Background—We tested the hypothesis that nuclear factor- κ B (NF- κ B) activity contributes to vascular endothelial dysfunction with aging and obesity in humans.

Methods and Results—We conducted a randomized, double-blind, placebo-controlled crossover study in 14 nondiabetic overweight or obese (body mass index ≥ 25 kg/m²) middle-aged and older (age 52 to 68 years) adults. Salsalate (nonacetylated salicylate, 4500 mg/d), a compound that inhibits NF- κ B activity, or placebo was administered for 4-day periods. Plasma salicylate concentrations reached the midtherapeutic range (21.8 ± 1.1 mg/100 mL, $P \leq 0.0001$ versus placebo) by day 4 of salsalate treatment. Salsalate increased expression of the inhibitor of NF- κ B and reduced total and nuclear expression of NF- κ B in endothelial cells obtained from the subjects (all $P < 0.05$). Salsalate increased brachial artery flow-mediated dilation by 74% (from $4.0 \pm 0.4\%$ to $6.6 \pm 0.5\%$, $P < 0.001$) but did not affect endothelium-independent dilation ($P = 0.83$). The change in brachial artery flow-mediated dilation with salsalate was inversely related to baseline flow-mediated dilation ($r = -0.77$, $P < 0.01$). Infusion of vitamin C increased brachial artery flow-mediated dilation during placebo ($P < 0.001$) but not after salsalate ($P = 0.23$). Salsalate reduced nitrotyrosine ($P = 0.06$) and expression of NADPH oxidase p47^{phox} ($P < 0.05$) in endothelial cells obtained from the subjects but did not influence circulating or endothelial cell inflammatory proteins.

Conclusions—Our findings provide the first direct evidence that NF- κ B, in part via stimulation of oxidative stress, plays an important role in mediating vascular endothelial dysfunction in overweight and obese middle-aged and older humans. (*Circulation*. 2009;119:1284-1292.)

Key Words: endothelium ■ brachial artery ■ obesity ■ inflammation ■ aging

Vascular endothelial dysfunction plays an important role in the increased risk of cardiovascular diseases with aging,¹ particularly in overweight and obese adults.^{2,3} Inflammation and oxidative stress are thought to be important processes by which aging and increased adiposity lead to development of vascular endothelial dysfunction⁴⁻⁷; however, the molecular mechanisms involved, particularly in humans, are poorly understood.

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The nuclear transcription factor nuclear factor- κ B (NF- κ B) and its inhibitory protein, inhibitor of NF- κ B (I κ B), compose a tightly controlled system that regulates the inflammatory and redox states of vascular endothelial cells.^{8,9} Data from animal and cell culture models implicate NF- κ B in both age- and obesity-associated vascular endothelial dysfunction.^{10,11} Recently, we found that endothelial cells obtained from overweight/obese¹² and older^{13,14} nondiabetic adults have greater

protein expression of NF- κ B than normal-weight and young adults, respectively. Moreover, NF- κ B was positively related to nitrotyrosine, a cellular marker of oxidative stress.¹³ Endothelial cell expression of the oxidant-producing enzyme NADPH oxidase also was greater in overweight/obese¹² and older¹³ adults. Taken together, these observations suggest that vascular endothelial dysfunction in middle-aged and older adults with increased body fat may be mediated in part by NF- κ B activation, perhaps through the development of oxidative stress; however, no direct evidence supports this concept in humans.

In the present study, we tested this hypothesis using a randomized, double-blind, placebo-controlled crossover design in nondiabetic overweight/obese middle-aged and older adults. Salsalate (nonacetylated salicylate), a compound that inhibits NF- κ B activation by preventing phosphorylation of I κ B and thus translocation of the p50/65 subunit of NF- κ B to the nucleus,^{9,15-17} or placebo was administered for 4-day

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periods. Endothelium-dependent (brachial artery flow-mediated dilation [FMD]) and endothelium-independent dilation were determined at the end of salsalate and placebo administration, and endothelial cells were obtained for subsequent analysis of I κ B, total and nuclear NF- κ B, and other inflammation-modulating proteins. Serum was assayed for metabolic risk factors, markers of inflammation and prostaglandin metabolism, and circulating vasoconstrictor factors. In a subset of the subjects, supraphysiological intravenous infusion of ascorbic acid (vitamin C) was used to assess oxidative stress-associated suppression of endothelium-dependent dilation under conditions of normal (placebo) and reduced (salsalate) NF- κ B activation. Endothelial cells were analyzed for nitrotyrosine and NADPH oxidase.

Methods

Subjects

Fourteen middle-aged and older (age 52 to 68 years) overweight and obese (body mass index ≥ 25 kg/m²) men and women were studied. Subjects were nonsmokers and nondiabetic and were free of other clinical diseases as assessed by medical history, physical examination, blood chemistry, and resting and exercise ECGs. Subjects were excluded if they were taking any prescription medications, herbal supplements, antioxidants, or aspirin. Subjects had elevated abdominal girth (waist circumference ≥ 94 cm for men and ≥ 80 cm for women, 12 of 14 subjects) and/or plasma C-reactive protein concentrations ≥ 1 to <10 mg/L (9 of 14) and 1 or more of the following: Hypertriglyceridemia (≥ 150 mg/dL, 3 of 14), low high-density lipoprotein cholesterol (<40 mg/dL in men and <50 mg/dL in women; 5 of 14), prehypertension (systolic blood pressure 130 to 139 mm Hg or diastolic blood pressure 85 to 89 mm Hg; 4 of 14), or high fasting glucose (100 to 125 mg/dL; 2 of 14). Subjects had performed no regular exercise (ie, <30 min/d, <2 days per week) for at least the prior 2 years. All procedures were approved by the Human Research Committee of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation.

Measurements

All measurements were performed at the University of Colorado at Boulder General Clinical Research Center after a 12-hour overnight fast and 24-hour abstinence from alcohol.

Subject Characteristics

Body mass index was calculated from height and weight to the nearest 0.1 kg, and waist and hip circumferences were measured by anthropometry.¹⁸ Total body fat was determined with dual-energy x-ray absorptiometry (DPX-IQ, GE/Lunar, Inc, Madison, Wis).¹⁹ Arterial blood pressure was measured during supine rest with a semiautomated device (Dynamap XL, Johnson and Johnson, Tampa, Fla), and maximal oxygen consumption ($\dot{V}O_{2\max}$), a measure of maximal aerobic exercise capacity, was determined as described previously.²⁰

Blood Analyses

Details of blood analyses can be found in the online-only Data Supplement.

Endothelial Cell Protein Expression

The procedures used for collection of venous endothelial cells and measurement of protein expression by quantitative immunofluorescence have been described in detail previously by our laboratory.^{12–14,21} Two sterile J wires (Daig Corp, Minnetonka, Minn) were advanced into an antecubital vein (≈ 4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter. The wires were then transferred to a dissociation buffer solution, and cells were recovered after a washing

and centrifugation protocol. Collected cells were fixed with 3.7% formaldehyde and plated on poly-L-lysine-coated slides (Sigma Chemical, St. Louis, Mo) and then frozen at -80°C until analysis.

Cells were rehydrated, and nonspecific binding sites were blocked with 5% donkey serum (Jackson ImmunoResearch, West Grove, Pa). Cells were incubated with monoclonal antibodies for NF- κ B p65, I κ B α (both from Novus, Littleton, Colo), nitrotyrosine, NADPH oxidase p47^{phox}, tumor necrosis factor (TNF)- α , monocyte chemoattractant protein-1 (MCP-1; all from Abcam, Cambridge, Mass), and cyclooxygenase (COX 1/2; Cayman Chemical, Ann Arbor, Mich). Cells were then incubated with an Alexafluor 555 fluorescent secondary antibody (Invitrogen Corp, Carlsbad, Calif).

For analysis, slides were viewed with a fluorescence microscope (Eclipse 600, Nikon, Melville, NY), and cell images were captured digitally by a Photometrics CoolSNAPfx digital camera (Roper Scientific, Inc, Tucson, Ariz). Endothelial cells were identified by staining for von Willebrand factor, and nuclear integrity was confirmed with DAPI (4',6'-diamidino-2-phenylindole hydrochloride). Once endothelial cells with intact nuclei were identified, they were analyzed with Metamorph Software (Universal Imaging Corp, Downingtown, Pa). For an estimate of nuclear NF- κ B p65, the nuclear regions of endothelial cells were identified by DAPI staining, overlaid on the same cell's Alexafluor 555 image and analyzed for fluorescent intensity of the Alexafluor within the DAPI region. Values for each protein are reported as a ratio of endothelial cell to human umbilical vein endothelial cell average pixel intensity. The technician was blinded to the identity of the subject and the experimental condition during the staining and analysis procedures. The reproducibility of our measurements of total and nuclear NF- κ B and I κ B α was determined recently in 4 subjects in whom endothelial cells were collected at least 1 week apart.¹⁴ Reproducibility was defined as the absolute difference in endothelial cell mean fluorescent intensity in trial 1 versus trial 2 divided by average mean fluorescent intensity for the trials. Mean values (range) were as follows: total NF- κ B 6.3% (4% to 8%), nuclear NF- κ B 7.1% (2% to 11%), and I κ B α 6.8% (0.4% to 15%).

Brachial Artery FMD and Endothelium-Independent Dilation

Brachial artery FMD and endothelium-independent dilation in response to 0.4 mg of sublingual nitroglycerin were determined with duplex ultrasonography (Powervision 6000, Toshiba, Inc, New York, NY) with a linear-array transducer as described previously by our laboratory.^{13,21–24} Details can be found in the online-only Data Supplement.

Study Design and Salsalate Administration

Subjects were randomly assigned oral doses of either salsalate or placebo for 4 consecutive days by use of a randomized crossover design. They received a standardized research diet prepared by the General Clinical Research Center bionutritionist for 3 days before each of the 2 vascular assessment sessions. Both subjects and investigators were blind to treatment condition. Salsalate was administered in a total daily dose of 4500 mg, with 2250 mg in the morning (taken in the General Clinical Research Center) and 2250 mg in the evening (at home). This dosing regimen has been used clinically to inhibit NF- κ B by producing steady state plasma salicylate concentrations in the therapeutic range of 10 to 30 mg/100 mL^{17,25,26} while minimizing gastrointestinal and other side effects.^{27,28} The treatment duration was chosen because ≈ 72 hours is required to attain maximal plasma concentrations of salicylate.^{29,30} Plasma salicylate concentrations were determined during the morning of days 0, 2, and 4 of salsalate administration to ensure concentrations were below toxic levels (<30 mg/100 mL). Subjects were telephoned in the evenings to remind them to take the second dose and to monitor them for any signs or symptoms of toxicity (eg, tinnitus, nausea, or rash). If subjects reported any of these signs or symptoms, they were instructed to not take the evening dose and to have plasma levels measured in the morning.

Seventeen subjects were enrolled in the experimental protocol. Three subjects withdrew during the protocol because of dizziness

Table 1. Baseline Subject Characteristics

	Mean±SE	Range
n	14	...
Sex, male/female, n	11/3	...
Age, y	61±1	52–68
Weight, kg	92±4	82–131
Body mass index, kg/m ²	31±2	26–42
Waist circumference, cm	101±4	80–127
Hip circumference, cm	109±3	101–135
Waist-hip ratio	0.94±0.03	0.74–1.09
Body fat, %	35±2	23–48
$\dot{V}O_2$ max, mL·kg ⁻¹ ·min ⁻¹	28±1	19–36

$\dot{V}O_2$ max indicates maximal exercise oxygen consumption.

(n=1) or tinnitus (n=1) on day 3 of salsalate treatment and development of gout during the placebo treatment (n=1). None of the 3 subjects had plasma salicylate concentrations in the toxic range.

Data Analysis

All data are presented as mean±SE. A paired *t* test was performed to determine differences in outcomes during salsalate versus placebo. Bivariate Pearson correlations were performed to assess relations of interest. Statistical significance was set a priori at *P*<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Baseline Status of Subjects

Baseline characteristics and serum factors are presented in Tables 1 through 3. Subjects were clinically overweight or obese, as indicated by a body mass index range of 26 to 42 kg/m², and had elevated abdominal adiposity based on waist circumference (Table 1). $\dot{V}O_2$ max ranged from 19 to 36 mL·kg⁻¹·min⁻¹, which reflects maximal aerobic exercise capacity within the low-normal range. Resting blood pressure and metabolic risk factors varied among individuals, but mean levels were within the clinically normal ranges (Table 2). Mean plasma C-reactive protein concentration (2.0±0.5 mg/L) was in the moderately elevated cardiovascular disease risk category (Table 3), consistent with chronic low-grade inflammation.³¹

Table 2. Blood Pressure and Circulating Metabolic Factors

	Placebo	Salsalate
Systolic blood pressure, mm Hg	124±3	118±3*
Diastolic blood pressure, mm Hg	72±2	69±2
Total cholesterol, mg/dL	205±12	180±8*
LDL cholesterol, mg/dL	130±9	113±10
HDL cholesterol, mg/dL	47±3	47±3
Triglycerides, mg/dL	139±18	81±11*
Glucose, mg/dL	95±2	84±2*

Values are mean±SE. LDL indicates low-density lipoprotein; HDL, high-density lipoprotein.

**P*<0.05 vs placebo.

Table 3. Humoral Factors

	Placebo	Salsalate
C-reactive protein, mg/L	2.0±0.5	2.2±0.4
Interleukin-6, pg/mL	1.8±0.2	1.3±0.2
TNF-α, pg/mL	1.4±0.2	1.5±0.1
Oxidized LDL, U/L	63±3	61±5
Leptin, ng/mL	11.3±2.3	13.1±3.0
Adiponectin, μg/mL	8.3±1.4	8.2±1.3
Endothelin-1, pg/mL	5.9±0.2	5.3±0.3
Norepinephrine, pg/mL	251±23	240±18
6-Keto prostaglandin F _{1α} , pg/mL	238±14	225±13
11-Dehydro thromboxane B ₂ , pg/mL	22.7±4.2	36.0±3.3*

Values are mean±SE. LDL indicates low-density lipoprotein.

**P*<0.05 vs placebo.

Oral Salsalate Increased Plasma Salicylate Concentrations to Therapeutic Levels

Plasma salicylate concentrations were undetectable at baseline, had increased by day 2 of salsalate administration (13.5±0.9 mg/100 mL, *P*<0.0001 versus baseline), and attained mean concentrations in the midtherapeutic range (21.8±1.1 mg/100 mL) by day 4 (*P*≤0.0001 versus baseline and day 2).

Salsalate Increased Expression of IκBα and Reduced Total and Nuclear NF-κB in Vascular Endothelial Cells

Compared with placebo, salsalate increased endothelial cell expression of IκBα (n=11) and reduced expression of total (n=13) and nuclear (n=13) NF-κB (all *P*<0.05; Figure 1A through 1C). Thus, salsalate treatment increased IκBα in endothelial cells from subjects in the present study, and this was associated with reduced NF-κB activation.

Salsalate Did Not Affect Expression of COX 1/2 in Vascular Endothelial Cells

Salsalate did not influence endothelial cell expression of COX 1/2 (n=10; *P*=0.50; Figure 1D). Plasma concentration of 6-keto-prostaglandin F_{1α} was not different (*P*=0.45), whereas plasma 11-dehydro thromboxane B₂ was increased (*P*<0.01) after salsalate (Table 3). These results suggest that salsalate had no effect on COX 1/2 expression in endothelial cells or on prostacyclin metabolism and may have increased thromboxane A₂ metabolism.

Salsalate Increased Endothelium-Dependent Dilation Without Affecting Endothelium-Independent Dilation

Compared with placebo, brachial artery FMD was 65% (percentage change in dilation) to 74% (absolute change, in millimeters of dilation) greater after salsalate treatment in the overall group (n=14; *P*<0.001; Figure 2A and 2B). In contrast, salsalate did not affect endothelium-independent dilation (n=11; *P*=0.83; Figure 2C and 2D). Brachial artery FMD was greater after salsalate in 13 of the 14 subjects (Figure 3A). The change in brachial artery

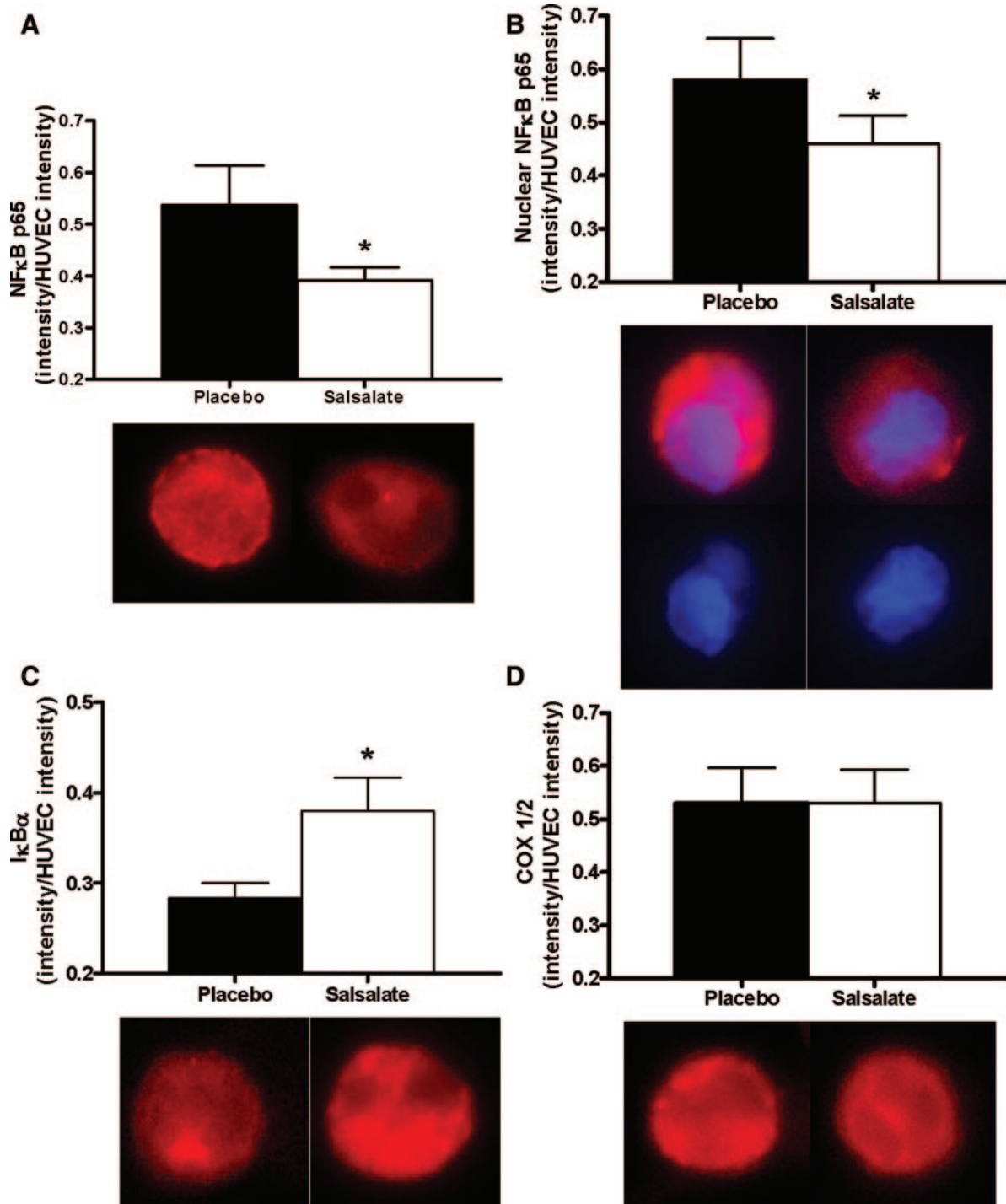


Figure 1. Protein expression of (A) whole-cell NF- κ B p65 ($n=13$), (B) nuclear NF- κ B p65 ($n=13$), (C) inhibitor of NF- κ B (I κ B α ; $n=11$), and (D) COX 1/2 ($n=10$) via quantitative immunofluorescent staining of endothelial cells obtained from an antecubital vein during placebo and salsalate conditions. Mean \pm SE values of fluorescent intensity adjusted for human umbilical vein endothelial cell (HUVEC) intensity are shown with examples of cells below each bar graph from individual subjects after placebo and salsalate. In B, DAPI nuclear staining (blue) overlay on whole-cell (red) NF- κ B p65 and DAPI stain alone are shown. * $P<0.05$ vs placebo.

FMD from placebo to salsalate treatment was strongly inversely related to baseline FMD ($n=14$; percentage change and absolute change in millimeters both $r=-0.77$, $P<0.01$; Figure 3B). Salsalate did not affect baseline or peak shear rate ($n=11$; both $P>0.05$ versus placebo). As such, FMD normalized for peak shear rate was 71% greater in the salsalate condition (0.0024 ± 0.0002 versus

0.0014 ± 0.0002 mm/s $^{-1}$, $P<0.001$). On the basis of a 2-tailed α -level of 0.05, the power achieved for determining differences in brachial FMD with the 14 subjects in the present study was 99%. These results demonstrate that salsalate selectively improved endothelium-dependent dilation, with the greatest improvements occurring in subjects with the most impaired baseline function.

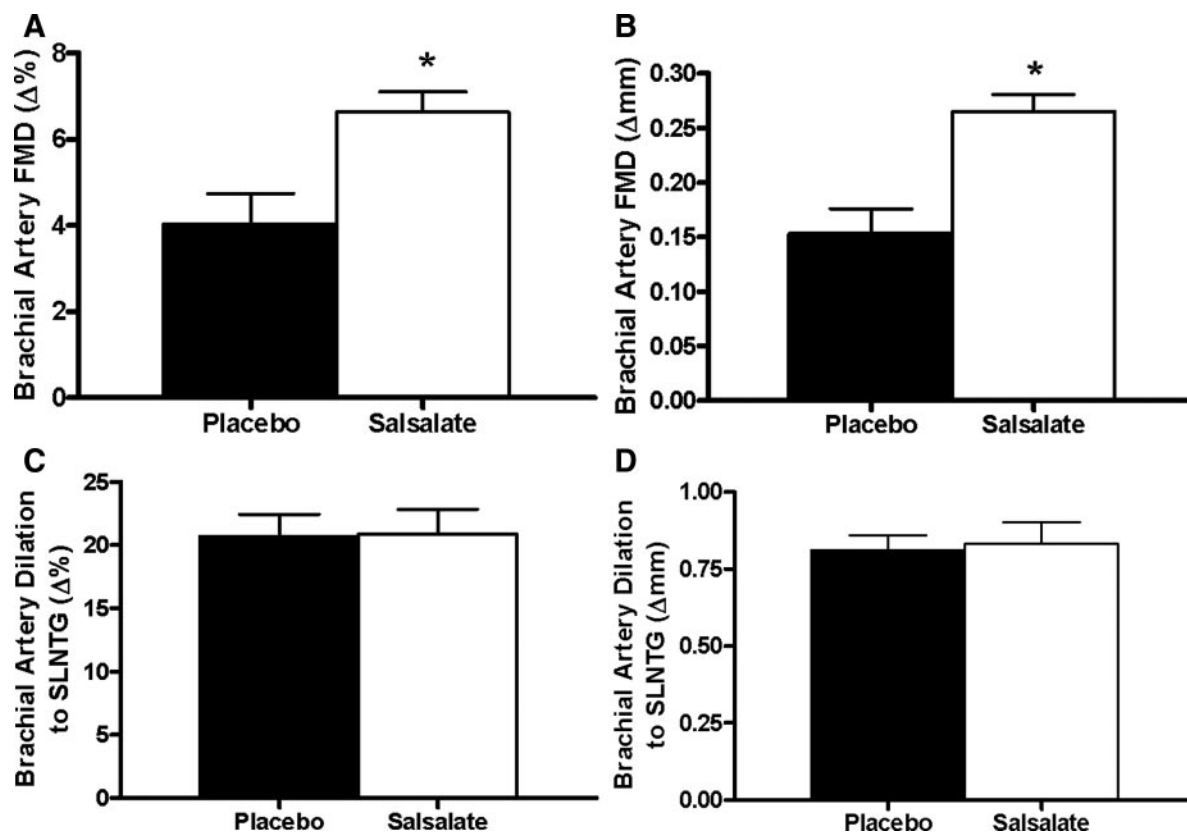


Figure 2. Endothelium-dependent dilation (n=14; brachial artery FMD; A, percentage change, $\Delta\%$; B, absolute change, Δmm) and endothelium-independent dilation (n=11; brachial artery dilation to sublingual nitroglycerin [SLNTG]; C, percentage change, $\Delta\%$; D, absolute change, Δmm) in subjects during placebo and salsalate conditions. Values are mean \pm SE. * $P < 0.05$ vs placebo.

Salsalate Abolished Oxidative Stress Suppression of Endothelium-Dependent Dilation and Reduced NADPH Oxidase p47^{phox} and Nitrotyrosine in Vascular Endothelial Cells

Compared with the saline control, intravenous infusion of vitamin C increased brachial artery FMD by $\approx 40\%$ during the placebo condition ($P < 0.001$; Figure 4, left). In contrast, vitamin C infusion had no effect on FMD after salsalate treatment ($P = 0.23$; Figure 4, right). Compared with the placebo condition, expression of NADPH oxidase p47^{phox} (n=13; $P < 0.05$) and nitrotyrosine (n=12; $P = 0.06$) was reduced in endothelial cells obtained from the subjects after salsalate treatment (Figure 5A and 5B). These observations indicate that salsalate abolished oxidative stress–associated inhibition of endothelium-dependent dilation and that this was associated with reduced expression of NADPH oxidase and decreased oxidative modifications in vascular endothelial cells.

Salsalate Did Not Influence Inflammatory Cytokines in Vascular Endothelial Cells

Compared with placebo, endothelial cell protein expression of TNF- α (n=11; placebo 0.67 ± 0.05 versus salsalate 0.63 ± 0.04 , TNF- α intensity/human umbilical vein endothelial cell intensity; $P = 0.25$) and MCP-1 (n=10; placebo 0.25 ± 0.03 versus salsalate 0.24 ± 0.03 MCP-1 intensity/human umbilical vein endothelial cell intensity; $P = 0.31$) was not affected by salsalate.

Salsalate-Associated Improvements in Endothelium-Dependent Dilation Were Not Related to Other Factors at Baseline or in Response to Salsalate

Resting systolic blood pressure and fasting plasma total cholesterol and glucose were 5% to 12% lower and plasma triglyceride concentrations were $\approx 42\%$ lower after salsalate treatment compared with the placebo condition (all $P < 0.05$; Table 2). Circulating low-density lipoprotein and high-density lipoprotein cholesterol (Table 2), markers of inflammation and oxidative stress, and vasoactive proteins (Table 3) were not different after salsalate (all $P > 0.05$ versus placebo), although plasma interleukin-6 tended to be lower ($P = 0.07$). The changes in brachial FMD with salsalate were not related to subject characteristics, blood pressure or circulating factors at baseline, changes in plasma salicylate concentrations, or any other factor in response to salsalate administration (all $P > 0.05$).

Discussion

On the basis of data from cell culture, animal models, and analysis of atherosclerotic plaques, NF- κ B is thought to play an important role in mediating endothelial dysfunction and increased risk of vascular diseases.^{10,32–35} To the best of our knowledge, however, no direct in vivo evidence exists in humans that NF- κ B contributes to chronically impaired vascular endothelial function with aging or obesity.

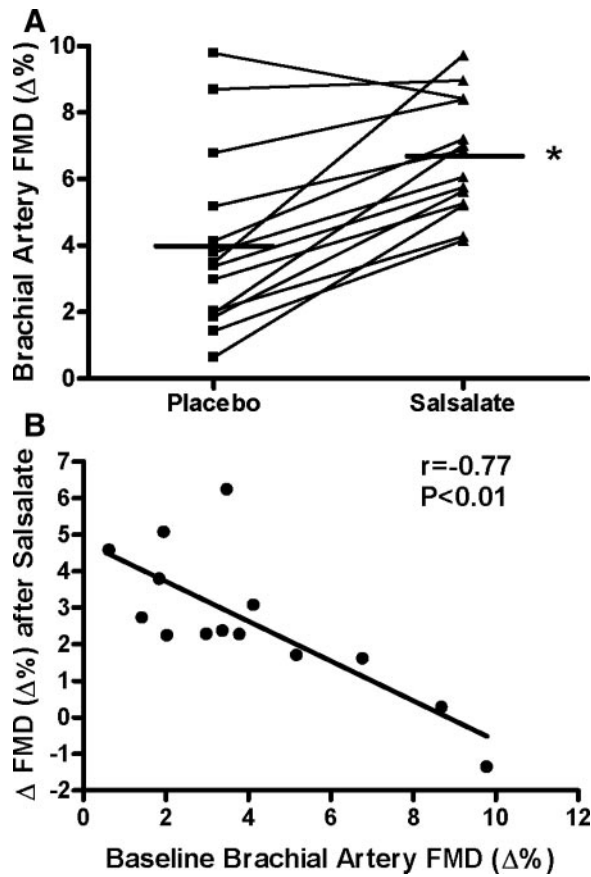


Figure 3. A, Individual values for endothelium-dependent dilation ($n=14$; brachial artery FMD; percentage change, $\Delta\%$) in subjects after placebo and salsalate conditions; B, Relation between baseline (placebo condition) brachial artery FMD and the change (Δ) in FMD from placebo to salsalate conditions. $*P < 0.05$ vs placebo.

In the present study, we tested this hypothesis in a group of overweight and obese middle-aged and older adults who had elevated abdominal adiposity and at least 1 other risk factor for cardiovascular disease. We found that short-term treatment with therapeutic doses of the NF- κ B inhibitor salsalate increased expression of I κ B α and reduced both total and nuclear NF- κ B in endothelial cells obtained from subjects in

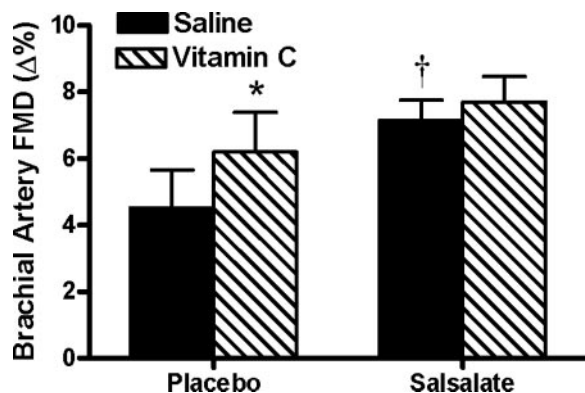


Figure 4. Endothelium-dependent dilation ($n=8$; brachial artery FMD) during intravenous infusion of saline and ascorbic acid (vitamin C) during placebo and salsalate conditions. Values are mean \pm SE. $*P < 0.05$ vs saline. $\dagger P < 0.05$ vs placebo.

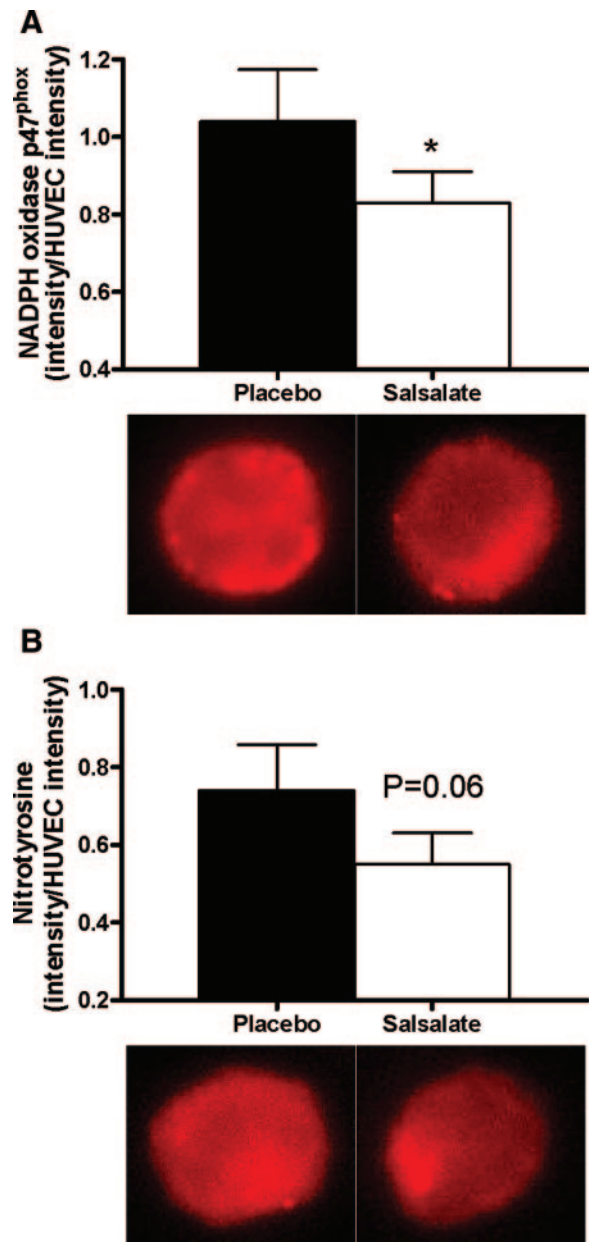


Figure 5. A, Protein expression of NADPH oxidase p47^{phox} ($n=13$) and (B) nitrotyrosine abundance ($n=12$) via quantitative immunofluorescent staining of endothelial cells obtained from an antecubital vein during placebo and salsalate conditions. Mean \pm SE values of fluorescent intensity adjusted for human umbilical vein endothelial cell (HUVEC) intensity are shown with examples of cells below each bar graph from individual subjects after placebo and salsalate. $*P < 0.05$ vs placebo.

the present study. The inhibition of NF- κ B by salsalate was associated with mean improvements in brachial artery FMD of $\approx 65\%$ to 75% . The effect was selective for the vascular endothelium, because endothelium-independent dilation was unchanged. Importantly, the improvement in brachial FMD with salsalate was observed in all but 1 subject and was strongly inversely related to baseline function, which indicates that NF- κ B-mediated suppression of endothelium-dependent dilation was greatest in the subjects with the most marked impairments in baseline vascular function. Infusion of supraphysiological concentrations of the antioxidant ascor-

bic acid improved brachial FMD during placebo control but had no effect during salsalate treatment, which indicates that NF- κ B suppresses endothelium-dependent dilation at least in part via oxidative stress. Consistent with this notion, in endothelial cells obtained from subjects in the present study, salsalate reduced expression of the p47^{phox} subunit of the oxidant-producing enzyme NADPH oxidase, as well as the abundance of nitrotyrosine, a cellular marker of oxidative modification of proteins. Finally, improvements in FMD with salsalate were not significantly related to any other baseline measure or to changes in any factor in response to treatment. Taken together, the present findings provide the first direct evidence in humans that activation of NF- κ B contributes to vascular endothelial dysfunction in nondiabetic middle-aged and older adults with elevated total and abdominal body fatness and chronic low-grade inflammation.

The present findings agree in part with the results of a recent nonrandomized, noncontrolled pilot intervention study in a small group of human immunodeficiency virus–infected patients (mean age 38 years) in whom brachial FMD was significantly increased after 8 but not 4 weeks of salsalate treatment (1500 mg twice daily).³⁶ Salsalate administration did not affect plasma markers of inflammation, including C-reactive protein, interleukin-6, soluble TNF- α receptor 1, MCP-1, or adhesion molecules. Consistent with these findings, in the present study, salsalate had no significant effects on plasma concentrations of C-reactive protein, interleukin-6, TNF- α , leptin, or adiponectin. Together, the results suggest that inhibition of NF- κ B with salsalate improves endothelium-dependent dilation in groups with impaired baseline function in the absence of clear reductions in circulating markers of inflammation.

Extensive *in vitro* evidence indicates that nonacetylated salicylic acids exert their cellular effects by inhibiting phosphorylation of I κ B, thus preventing the translocation of NF- κ B to the nucleus.^{9,15,16} In agreement, here we present *in vivo* evidence that short-term systemic administration of salsalate increases I κ B α expression in endothelial cells from human subjects with impaired baseline endothelial function. The increase in I κ B α was associated with reduced nuclear expression of NF- κ B and a marked increase in endothelium-dependent dilation. The latter findings are consistent with previous observations from our laboratory that NF- κ B expression is increased in endothelial cells from overweight/obese¹² and older^{13,14} adults and is inversely related to brachial artery FMD.¹³

The results of the present study also provide insight into the mechanisms by which NF- κ B inhibition improves vascular endothelial function in this group. Excessive bioavailability of reactive oxygen species (oxidative stress) suppresses endothelium-dependent dilation in settings of increased adiposity and aging.^{7,23,37} In turn, NF- κ B stimulates the production of reactive oxygen species at least in part via activation of NADPH oxidase.^{38,39} As such, we reasoned that inhibition of NF- κ B by salsalate might improve endothelium-dependent dilation by reducing oxidative stress. In support of this, we found that intravenous infusion of ascorbic acid (vitamin C) improved brachial artery FMD under placebo conditions but not after salsalate treatment. Moreover, the fact that nitroty-

rosine was reduced in endothelial cells obtained from subjects in the present study after administration of salsalate provides molecular evidence for reduced vascular endothelial oxidative stress after treatment. Salsalate also reduced NADPH oxidase, which suggests that reduced expression of this oxidant-producing enzyme could be 1 mechanism contributing to the decrease in oxidative stress. Together, the present functional and molecular data are consistent with the idea that NF- κ B activation is associated with an upregulation of NADPH oxidase and oxidative stress *in vivo* in middle-aged and older adults with increased body fatness, and this, in turn, contributes to impaired endothelial function. However, reactive oxygen species also can activate NF- κ B.^{10,40} The present data do not provide insight as to the direction of the relation between these events.

In addition to modulating redox signaling in the cell, NF- κ B facilitates transcription of a large number of proinflammatory genes.⁴¹ As a result, NF- κ B also may suppress endothelium-dependent dilation via increased proinflammatory signaling. In the present study, salsalate treatment did not influence plasma inflammatory markers. Insufficient numbers of endothelial cells were available from our technique to measure a wide array of inflammatory proteins in addition to those required to test our formal hypotheses. However, expression of the proinflammatory cytokines TNF- α and MCP-1 did not differ in the salsalate and placebo conditions. Thus, although it is possible, we have no direct evidence that NF- κ B inhibited endothelium-dependent dilation through modulation of inflammatory signaling independent of oxidant effects.

Salsalate (ie, sodium salicylate) is an aspirin-like compound, but it lacks an acetyl group needed to directly inhibit COX-mediated production of prostaglandins.^{17,42} In the present study, we established that salsalate treatment did not affect COX 1/2 expression in vascular endothelial cells obtained from the subjects or plasma concentration of 6-keto-prostaglandin F_{1 α} , a marker of prostacyclin metabolism. Plasma 11-dehydro thromboxane B₂ was greater after salsalate, which suggests that thromboxane A₂ metabolism may have been increased. Together, these data indicate that salsalate did not inhibit COX. Indeed, salsalate produced an improvement in FMD despite a possible increase in circulating thromboxane, which has vasoconstrictor effects.⁴³

We measured blood pressure and a variety of circulating factors that could have changed with salsalate treatment and influenced vascular function. Salsalate did not affect the majority of these factors. However, systolic blood pressure, plasma total cholesterol, and fasting glucose were modestly reduced, and a marked reduction in fasting plasma triglycerides was observed after only 4 days of salsalate. These effects were not a result of changes in diet, because subjects were placed on an identical research diet for 3 days before each experimental visit, thus ensuring similar total energy and macronutrient intake during the placebo and salsalate conditions (online-only Data Supplement Table I). The improvements in brachial FMD with salsalate were not related to baseline levels or treatment-related changes in any factor; however, it is possible that such relations existed but were not evident because of the small number of subjects. Therefore,

we cannot rule out the possibility that the reductions in these selective risk factors contributed to improvements in FMD with salsalate.

Because the acquisition of arterial endothelial cells would have required invasive arterial catheterization twice (placebo and salsalate conditions) over a short period of time, venous sampling was used in the present study. However, we^{13,14} and others^{44,45} have shown that group- and treatment-associated differences in expression of NF- κ B, COX, and other proteins can be assessed in endothelial cells obtained from peripheral veins. Moreover, salsalate was administered systemically, and we were able to show the expected effects of this compound on expression of I κ B α and total and nuclear NF- κ B in endothelial cells obtained from antecubital veins of our subjects.

We believe that the present findings have important clinical relevance. Overweight and obese middle-aged and older adults constitute a significant portion of the population at elevated risk of cardiovascular disease. Vascular endothelial dysfunction is considered a central feature of clinical vascular disorders. Despite this, the cellular and molecular mechanisms that contribute to impaired endothelial function in humans are incompletely understood. The present results provide unique and direct in vivo evidence that supports a key role for the transcription factor NF- κ B in mediating vascular endothelial dysfunction in humans and provide insight into the underlying mechanisms. Inhibition of NF- κ B with salsalate improved brachial artery FMD in study subjects from $4.0 \pm 0.4\%$ to $6.6 \pm 0.5\%$. By comparison, using the same method, we have found that brachial FMD averages 7.2% in young adult control subjects studied in our laboratory (sample $n=62$, 47 males and 15 females, 22.7 ± 0.4 years of age). Thus, our results suggest that as much as 80% of the impairment in brachial artery FMD was restored by short-term treatment with salsalate. Although we cannot definitively attribute this entire effect to inhibition of NF- κ B, the present results strongly implicate NF- κ B activation and its associated mechanisms of action.

In summary, the present findings provide the first direct evidence in humans that NF- κ B, in part via stimulation of oxidative stress, plays an important role in mediating endothelial dysfunction in peripheral conduit arteries. Our results provide further support for the concept that inhibition of NF- κ B may be an effective therapeutic strategy in the prevention and treatment of age- and obesity-related vascular endothelial dysfunction in humans.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Middle-aged and older overweight and obese adults are at elevated risk of cardiovascular disease. This is attributable in part to vascular endothelial dysfunction, as indicated by impaired endothelium-dependent dilation; however, the cellular and molecular mechanisms involved are incompletely understood. The present study provides direct in vivo evidence that supports a key role for the proinflammatory, redox-sensitive transcription factor nuclear factor κ B (NF- κ B) in mediating vascular endothelial dysfunction in overweight/obese middle-aged and older adults. In 14 nondiabetic (body mass index ≥ 25 kg/m²) men and women 52 to 68 years of age (randomized, double-blind, placebo-controlled crossover study), 4 days of treatment with oral salsalate (nonacetylated salicylate, 4500 mg/d), an NF- κ B inhibitor, reduced total and nuclear NF- κ B in vascular endothelial cells and improved brachial artery flow-mediated dilation by 74%. Improvements in brachial artery flow-mediated dilation with salsalate were inversely related to baseline flow-mediated dilation ($r = -0.77$). Infusion of the antioxidant vitamin C improved brachial artery flow-mediated dilation during placebo but not after salsalate. Salsalate reduced nitrotyrosine, a marker of oxidative stress, and expression of the oxidant enzyme NADPH oxidase p47^{phox} in vascular endothelial cells. Salsalate also reduced systolic blood pressure and improved plasma lipids and glucose. The present findings suggest that NF- κ B, in part via stimulation of oxidative stress, plays an important role in mediating endothelial dysfunction in peripheral conduit arteries in humans. Our results provide support for the concept that inhibition of NF- κ B may be an effective therapeutic strategy in the prevention and treatment of age- and obesity-related vascular endothelial dysfunction.

DATA SUPPLEMENT

**Nuclear Factor- κ B Activation Contributes to Vascular Endothelial
Dysfunction via Oxidative Stress in Overweight/Obese Middle-Aged
and Older Humans**

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Supplemental Methods.

Blood Analyses. The main assays were performed by the University of Colorado Adult GCRC core laboratory. Fasting plasma total cholesterol, LDL-C, HDL-C, triglycerides and glucose were determined using standard assays. Plasma concentrations of CRP were measured using a high-sensitivity Chemistry Immuno Analyzer (AU400e, Olympus America, Inc.). Plasma interleukin-6 (IL-6), tumor-necrosis factor- α (TNF- α ; both R&D Systems, Inc), oxidized LDL (Alpco, Inc.), endothelin-1 (Peninsula Laboratories, Inc) were measured by ELISA. Serum adiponectin and leptin were measured by radioimmunoassay (Linco Research, Inc.) and plasma norepinephrine by high performance liquid chromatography. Plasma salicylate concentrations were determined by the Boulder Community Hospital Clinical Chemistry laboratory using a validated colormetric assay (Ortho Diagnostics, Inc.). Plasma concentrations of 6-keto-prostaglandin $F_{1\alpha}$ and 11-dehydro-thromboxane B_2 , major metabolites of prostacyclin and thromboxane A_2 , respectively, were measured by the clinical laboratory of Cayman Chemicals, Inc. (Ann Arbor, MI) using ELISA.

Brachial Artery FMD and Endothelium-Independent Dilation. Responses are expressed as mm and % maximal change from baseline diameter per recent recommendations ¹. Baseline and hyperemic blood flow data were available on 11 subjects. Because blood viscosity was not available to calculate shear stress, baseline shear rate was calculated as baseline blood flow velocity/baseline diameter and peak shear rate as mean peak blood flow velocity/occlusion diameter (i.e., average diameter for 15 seconds prior to release of occlusion cuff pressure) ². In a subgroup of 8

subjects, FMD was measured during saline (control) and during supraphysiological intravenous infusion of vitamin C (ascorbic acid) on the same day as described previously by our laboratory ³. Briefly, subjects received an intravenous saline bolus for 20 min followed by a 60 min saline drip infusion. Brachial artery FMD baseline measurements were obtained immediately after the saline bolus during the saline drip infusion. Next, an ascorbic acid priming bolus of 0.06 g/kg fat-free mass was infused for 20 minutes followed by a drip infusion of 0.02 g/kg fat-free mass administered over 60 minutes. Brachial artery FMD and endothelium-independent dilation were measured after the intravenous bolus of ascorbic acid during the drip infusion because peak plasma concentrations of ascorbic acid occur after the bolus infusion using this protocol ³.

Supplemental Results.

Supplemental Table I: Energy and macronutrient intake from 3-day research diet

	Placebo	Salsalate
Calories (kcal/day)	2382 ± 106	2316 ± 111
Carbohydrate (g/day)	333 ± 15	325 ± 16
Protein (g/day)	91 ± 4	90 ± 4
Fat (g/day)	82 ± 4	79 ± 4
Carbohydrate (% of kcal/day)	54.8 ± 0.2	54.6 ± 0.6
Protein (% of kcal/day)	15.0 ± 0.1	15.3 ± 0.2
Fat (% of kcal/day)	30.2 ± 0.2	30.1 ± 0.5

Values are mean ± standard error.

Supplemental References.

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