Evidence That Niacin Inhibits Acute Vascular Inflammation and Improves Endothelial Dysfunction Independent of Changes in Plasma Lipids

Ben J. Wu, Ling Yan, Francesca Charlton, Paul Witting, Philip J. Barter, Kerry-Anne Rye

Objective—To determine if niacin can confer cardiovascular benefit by inhibiting vascular inflammation and improving endothelial function independent of changes in plasma lipid and lipoprotein levels.

Methods and Results—New Zealand white rabbits received normal chow or chow supplemented with 0.6% or 1.2% (wt/wt) niacin. This regimen had no effect on plasma cholesterol, triglyceride, or high-density lipoprotein level. Acute vascular inflammation and endothelial dysfunction were induced in the animals with a periarterial carotid collar. At the 24-hour postcollar implantation, the endothelial expression of vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and monocyte chemotactic protein 1 was markedly decreased in the niacin-supplemented animals compared with controls. Niacin also inhibited intima-media neutrophil recruitment and myeloperoxidase accumulation, enhanced endothelial-dependent vasorelaxation and cyclic guanosine monophosphate production, increased vascular reduced glutathione content, and protected against hypochlorous acid–induced endothelial dysfunction and tumor necrosis factor α–induced vascular inflammation.

Conclusion—Previous human intervention studies have demonstrated that niacin inhibits coronary artery disease. This benefit is thought to be because of its ability to reduce low-density lipoprotein and plasma triglyceride levels and increase high-density lipoprotein levels. The present study showed that niacin inhibits vascular inflammation and protects against endothelial dysfunction independent of changes in plasma lipid levels. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: niacin • inflammation • endothelial dysfunction

Niacin (nicotinic acid) has been used for more than 30 years to treat plasma lipid disorders and to prevent atherosclerotic cardiovascular disease. At pharmacological doses, niacin reduces low-density lipoprotein cholesterol, plasma triglyceride, nonesterified fatty acid, and lipoprotein(a) levels. Niacin also increases the concentration of high-density lipoproteins (HDLs). Human intervention studies have indicated that treatment with niacin, either alone or in combination with other lipid-lowering agents, can slow or reverse the progression of atherosclerosis and reduce cardiovascular event rates and total mortality in patients with hypercholesterolemia and established atherosclerotic cardiovascular disease. In combination therapy with statins, niacin reduces cardiovascular events and slows coronary atherosclerosis progression. It also reduces coronary stenosis progression in patients with metabolic syndrome. It has always been assumed that these beneficial effects are the result of the lipid-modifying effects of niacin.

However, recent data have indicated that niacin also decreases C-reactive protein levels, improves endothelial dysfunction, increases the endothelial and leukocyte oxidation-reduction (re/dox) state in vitro, inhibits cytokine-induced monocyte adhesion to human endothelial cells, improves plaque stability, and reduces thrombosis. It also decreases cytokine-induced expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and monocyte chemotactic protein 1 (MCP-1) in cultured human endothelial cells. These results suggest that niacin may prevent cardiovascular disease by inhibiting vascular inflammation via mechanisms independent of its lipid-modifying effects.

This question was addressed in the present study by determining if niacin inhibits acute vascular inflammation and improves endothelial function in normocholesterolemic and non-atherosclerotic rabbits, an animal model in which niacin does not affect plasma lipids. The results establish that niacin inhibits acute vascular inflammation and protects against endothelial dysfunction independent of changes in plasma lipid levels.

Methods

New Zealand white (NZW) rabbits (n=6 to 9 per group) were fed regular chow or chow supplemented with 0.6% or 1.2% (wt/wt)
Niacin for 14 days, before the insertion of a nonocclusive periarterial carotid collar. The animals were euthanized 24 hours after collar insertion, when the carotid arteries were extracted and subjected to immunohistochemical analysis for expression of VCAM-1, ICAM-1, MCP-1, intima-media neutrophil content (CD18+ cells), and myeloperoxidase (MPO) levels. Ex vivo isometric tension experiments were performed to assess the effects of niacin on endothelial function in the collared and noncollared carotid arteries and the thoracic aortic rings. Thoracic aortic rings were also incubated in vitro in the presence or absence of niacin and hypochlorous acid (HOCl).

Results

Niacin Attenuates Vascular Adhesion Molecule and Chemokine Expression

Three groups of NZW rabbits (n=6 to 9 per group) were used for the study. Group 1 was fed regular chow (n=9). The other groups consumed chow supplemented with 0.6% (n=9) or 1.2% (n=6) niacin (wt/wt) for 2 weeks,12,13 after which plasma niacin levels were 38±17 μmol/L for the animals that received 0.6% niacin and 44±20 μmol/L for the animals that received 1.2% niacin.

There were no significant differences in plasma total cholesterol, HDL cholesterol, triglyceride, nonesterified fatty acid, and apolipoprotein A-I levels between the groups when measured before niacin treatment and when euthanized (Supplemental Table; all supplemental material available online at http://atvb.ahajournals.org).

The implantation of a periarterial carotid collar did not affect endothelial morphological features, as assessed by immunohistochemical staining for CD31 (Supplemental Figure I). As previously reported, the collar increased the endothelial expression of VCAM-1, ICAM-1, and MCP-1 in noncollared arteries (open bars) and collared arteries (closed bars). There were no significant differences in plasma total cholesterol, HDL cholesterol, triglyceride, nonesterified fatty acid, and apolipoprotein A-I levels between the groups when measured before niacin treatment and when euthanized (Figure 1).

The implantation of a periarterial carotid collar did not affect endothelial morphological features, as assessed by immunohistochemical staining for CD31 (Supplemental Figure I). As previously reported, the collar increased the endothelial expression of VCAM-1, ICAM-1, and MCP-1.14 (Figure 1). At 24 hours after collar implantation, endothelial VCAM-1 expression had increased from 0.3±0.1 to 4.8±0.6 image units (P<0.001). ICAM-1 expression increased from 1.0±0.2 to 6.4±0.4 image units (P<0.001), and MCP-1 expression increased from 0.2±0.1 to 6.0±1.1 image units (P<0.001) (Figure 1A and B). VCAM-1, ICAM-1, and MCP-1 mRNA levels increased 2.8-, 10.1-, and 19.8-fold, respectively (P<0.05) (Figure 1C).

Relative to the collared animals that received regular chow, dietary supplementation with 0.6% and 1.2% niacin de-
increased the endothelial expression of VCAM-1 by 55±10% and 52±16%, respectively; ICAM-1 expression by 48±10% and 44±13%, respectively; and MCP-1 expression by 77±8% and 83±14%, respectively (P<0.05 for all) (Figure 1A and B). Supplementation with 0.6% and 1.2% niacin also decreased VCAM-1 mRNA levels by 53±3% and 45±8%, respectively; ICAM-1 mRNA levels by 66±18% and 72±13%, respectively; and MCP-1 mRNA levels by 91±4% and 82±10%, respectively (P<0.05 for all) (Figure 1C).

Niacin Inhibits Neutrophil Infiltration and MPO Accumulation in the Intima-Media

The collar mediated intima-media neutrophil infiltration, with the area of CD18+ staining increasing from 1.0±0.1% in the noncollared arteries to 18.1±5.1% in the collared arteries (P<0.01) (Figure 2A and B): Intima-media MPO levels were elevated in the collared arteries, as indicated by an increase in staining from 0.9±0.1% to 20.1±4.8% (P<0.01) (Figure 2A and B). This indicates that collar implantation could potentially lead to accumulation of neutrophil-derived reactive oxygen species (ROS). These collar-induced inflammatory responses were inhibited by niacin. Supplementation with 0.6% and 1.2% niacin inhibited neutrophil recruitment into the vessel wall by 86±7% and 89±5%, respectively (P<0.05); and reduced MPO levels by 73±7% and 81±6%, respectively (P<0.05) (Figure 2A and B). Across the collared arteries in all 3 groups of animals (n=18), neutrophil recruitment into the intima-media correlated positively with MPO accumulation (r=0.95, P<0.001) (Figure 2C), indicating that the neutrophils were activated and released MPO. Because the total blood neutrophil counts did not differ significantly between groups (Supplemental Figure II), this result could not be attributed to niacin reducing the number of circulating neutrophils.

Niacin Increases Vascular Total Glutathione Content

It was previously reported that periarterial collars increase ROS production in the vessel wall. Reduced glutathione (GSH) is the principal intracellular nonprotein thiol that scavenges ROS, and niacin has been reported to increase the GSH content of cultured human aortic endothelial cells. The results in Figure 3 establish that this is also the case in vivo. The total GSH content of the noncollared carotid arteries from the animals that received 0.6% and 1.2% niacin increased by 2.2±0.6-fold (P<0.05) and 2.9±0.7-fold (P<0.01), respectively, compared with control arteries (Figure 3A, open bars). In contrast, the total GSH content of the collared arteries from the control and the niacin-treated animals (Figure 3A, closed bars) was decreased by 90% compared with the noncollared arteries. This is consistent with the GSH in these arteries being depleted by the ROS generated by the activated neutrophils.

There was also a 2.1±0.4- and a 2.3±0.5-fold increase in the total GSH content of the thoracic aorta from the animals that were supplemented with 0.6% and 1.2% niacin, respectively, compared with control (P<0.05) (Figure 3B). Ex vivo incubation with 1-mmol/L niacin increased the GSH content of control thoracic aortas by 2.9±0.5-fold (P<0.05). Incubation with 0.5-mmol/L niacin did not significantly increase the thoracic aorta GSH content (Figure 3C).

Niacin Inhibits Cytokine-Induced Inflammation in the Rabbit Thoracic Aorta

Niacin inhibits tumor necrosis factor α (TNF-α)–induced inflammation in cultured human endothelial cells. To determine if this is also the case in vivo, thoracic aortic segments from control rabbits were incubated for 6 hours with TNF-α. This increased the VCAM-1 mRNA level by...
segments stimulated with TNF-α and incubated without niacin, incubation with 1-mmol/L niacin decreased VCAM-1, ICAM-1, and MCP-1 mRNA levels by 58.0±8.9%, 39.0±6.1%, and 45.0±11.0%, respectively (P<0.05) (Figure 4B). Incubation with human endothelial cell growth medium serum-free culture medium alone did not change VCAM-1, ICAM-1, and MCP-1 mRNA levels in control thoracic aortic segments (Supplemental Figure III).

**Niacin Protects Against Collar-Induced Endothelial Dysfunction**

ROS, such as HOCl, and cytokines, such as TNF-α, which are produced by activated leukocytes under conditions of acute vascular inflammation, can induce endothelial dysfunction and decrease endothelial-derived nitric oxide bioavailability. This decreases intracellular cyclic guanosine monophosphate (cGMP) production and reduces vasorelaxation in response to endothelial-derived nitric oxide agonists, such as acetylcholine. The implantation of periartrial collars in rabbits is known to impair endothelium-dependent vasorelaxation. This was confirmed in the present study, with acetylcholine-mediated endothelium-dependent vasorelaxation in carotid artery rings from collared rabbits being significantly reduced by 24-hour postcollar implantation (Figure 5A, closed triangles) compared with rings from noncollared carotid arteries (Figure 5A, open triangles) (P<0.001). Relative to what was observed for collared animals that did not receive niacin, collared carotid artery rings from rabbits that received 0.6% niacin (Figure 5A, closed circles) displayed increased vascular relaxation (P<0.01) (Figure 5A).

The cGMP content in response to acetylcholine of collared arteries from animals that did not receive niacin was also decreased by 87.0±3.2% compared with that of noncollared arteries (P<0.001) (Figure 5B). Relative to what was observed for collared arteries from chow-fed animals, dietary supplementation with 0.6% niacin increased the cGMP content of the collared arteries by 2.9±0.5-fold (P<0.05) (Figure 5B). Collar implantation and treatment with niacin had no effect on sodium nitroprusside–induced endothelial-independent vasorelaxation (Figure 5C).

**Niacin Protects Against HOCl-Induced Endothelial Dysfunction**

The MPO that is generated by activated neutrophils consumes hydrogen peroxide, forming HOCl. Because HOCl contributes to endothelial dysfunction directly by inhibiting nitric oxide production, and because GSH is the principal scavenger of HOCl in endothelial cells, we hypothesized that the increased aortic GSH content in the rabbits that received niacin may prevent HOCl-induced endothelial dysfunction. When thoracic aortic rings from animals that did not receive niacin were preincubated with HOCl, acetylcholine-mediated vasorelaxation was essentially abolished (Figure 6A, open triangles). When, in contrast, aortic rings from animals that received 0.6% niacin were incubated with HOCl, relaxation was increased 6.1±1.4-fold at the highest concentration of acetylcholine (P<0.001) (Figure 6A, open circles). This is less than what was observed for the control (Figure 6A, open circles).
6A, closed triangles) and the niacin-treated aortic rings (Figure 6A, closed circles) that were incubated without HOCl.

The cGMP content in response to acetylcholine of aortic rings from chow-fed and niacin-supplemented animals was not affected by incubation with phosphate-buffered saline (Figure 6B, closed bars). When aortic rings from the control animals were incubated with HOCl, cGMP production in response to acetylcholine stimulation was minimal (Figure 6B, open bars), but increased by 4.1 ± 0.9-fold for aortic rings from the animals that received dietary supplementation with 0.6% niacin (P < 0.05) (Figure 6B, open bars).

A similar outcome was observed when thoracic aortic rings from animals that had not received niacin were incubated ex vivo with niacin. Compared with rings incubated with HOCl in the absence of niacin (Figure 6C, open triangles), incubation with niacin afforded partial protection from the HOCl-induced reduction in vasorelaxation in response to acetylcholine (P < 0.001) (Figure 6C, open circles). As was observed for thoracic aortic rings from niacin-treated animals, acetylcholine-stimulated relaxation of the rings incubated with HOCl and niacin was less than that of rings incubated without HOCl in the presence or absence of niacin (Figure 6C, closed symbols). The rings that were incubated with niacin in the absence of HOCl also had comparable cGMP contents in response to acetylcholine (Figure 6D, closed bars). The cGMP level in response to acetylcholine in rings incubated with niacin plus HOCl, in contrast, was 3.3 ± 1.0-fold (P < 0.05) greater than that of rings incubated with HOCl in the absence of niacin (Figure 6D, open bars).

**Discussion**

For the first time, to our knowledge, this report demonstrates that niacin markedly inhibits acute vascular inflammation and protects against endothelial dysfunction in normocholesterolemic and nonatherosclerotic rabbit arteries by processes independent of changes in plasma lipid and HDL levels. These beneficial effects of niacin were associated with an improved vascular redox state, which protected against ROS-induced endothelial dysfunction and inhibited cytokine-induced vascular inflammation.

A relationship between the anti-inflammatory properties of niacin and its ability to improve endothelial function has been demonstrated previously in human studies. For example, extended-release niacin decreased levels of C-reactive protein by 15% in patients with stable coronary artery disease.6 It also decreased C-reactive protein levels and improved endothelial function in subjects with metabolic syndrome.21 Improvement of endothelial function by extended-release niacin has also been reported in individuals with coronary artery disease.22 However, the anti-inflammatory effects and improved endothelial function that were observed in those studies could not be dissociated from the increased HDL levels, which can also mediate these effects.23 Evidence that niacin may have anti-inflammatory properties independent of its ability to increase HDL levels comes from a recent in vitro study9 in which TNF-α-induced VCAM-1 and MCP-1 expression and monocyte adhesion to cultured human aortic endothelial cells was significantly inhibited. Niacin also reduces ICAM-1 and PECAM-1 protein levels in TNF-α-activated human umbilical vein endothelial cells.26 The present results extend these findings by showing that these effects are also apparent in vivo and that they are independent of changes in plasma lipid levels.

Niacin is a precursor of nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate,24 and GSH, the principal intracellular nonprotein thiol responsible for maintaining intracellular redox states and protecting cells against oxidative stress.15 Ganji et al9 have shown that niacin increases nicotinamide adenine dinucleotide phosphate and GSH levels in vascular endothelial cells and decreases ROS formation and oxidative-related inflammatory responses. Consistent with these findings, we have shown that the total GSH content of aortic rings is increased by ex vivo incubation with niacin and by dietary niacin supplementation. These aortic rings were also protected against HOCl-induced endothelial dysfunction and cytokine-induced vascular inflammation.

GSH is a well-recognized scavenger of MPO-derived oxidants, such as HOCl,15 pathophysiological concentrations of which rapidly deplete intracellular GSH levels in cultured human umbilical vein endothelial cells.20,26 Consistent with
this finding, the present results show that the total GSH content of collared arteries from control and niacin-treated animals was essentially depleted at 24 hours after collar implantation (Figure 3A). This suggests that the increased GSH may have reduced collar-induced ROS production, and raises the possibility that niacin, as a source of GSH, could potentially protect against ROS-induced injury. Together, these results indicate that niacin may protect against carotid collar-induced vascular inflammation and improve endothelial function, at least in part, by increasing vascular GSH, which scavenges the ROS that is produced by neutrophil-generated MPO in the vessel wall.

The precise mechanism of the anti-inflammatory effects of niacin is not entirely understood. Direct involvement of the niacin receptor GPR109A is unlikely because it is not expressed in vascular tissues. However, neutrophils are known to express a functional form of the GPR109A receptor that may be important for mediating nicotinic acid–induced neutrophil apoptosis. This raises the possibility of indirect participation of GPR109A in the anti-inflammatory effects of niacin.

Activation of the transcription factor nuclear factor NFkB by cytokines, such as TNF-α, increases endothelial expression of VCAM-1, ICAM-1, and MCP-1; and is known to play an important role in the pathogenesis of atherosclerosis by recruiting circulating leukocytes into the vessel wall. Furthermore, NF-κB is redox sensitive and is activated by ROS, such as hydrogen peroxide, and ionizing radiation. Depletion of cellular GSH can also degrade the NF-κB inhibitory subunit. When taken together, these observations suggest that the ability of niacin to inhibit acute vascular inflammation in the present study may be related to inhibition of NF-κB activation. This is consistent with 2 recent reports showing that niacin reduces VCAM-1, ICAM-1, and MCP-1 expression in cultured endothelial cells by inhibiting NF-κB.

Although niacin has been used for more than 30 years to treat dyslipidemia in humans, the metabolic pathways it influences and the associated pharmacokinetics are not well established. In humans, niacin levels increase rapidly, peaking at approximately 1 hour after oral administration and returning to baseline by 2 hours after ingestion. Niacin is also metabolized to nicotinuric acid, which is cleared from the circulation at a much slower rate than niacin. As niacin was incorporated into chow to which the animals in the present study had unrestricted access, plasma niacin levels varied widely (Supplemental Figure IV). This indicates that simply measuring plasma niacin levels does not accurately reflect the overall niacin concentrations achieved during the study period. Niacin is also metabolized to nicotinic acid, which is cleared from the circulation at a much slower rate than niacin. Further investigation is required to give an insight into the bioavailability of niacin in vivo and to investigate whether its metabolites also have cardioprotective properties.

In the present study, niacin treatment tended to decrease plasma triglyceride and nonesterified fatty acid levels (Supplemental Table); however, these reductions did not reach statistical significance (P>0.10 for all; n=6). This indicates that the markedly reduced vascular inflammation and improved endothelial function that was observed in the niacin-treated animals cannot be attributed to changes in lipid profiles. Additional evidence that this is the case comes from the ex vivo study that showed that incubation with niacin protects against HOCl-induced endothelial dysfunction and cytokine-induced vascular inflammation in aortic rings from control animals that did not receive dietary supplementation.
with niacin (Figure 6). Niacin can also change the composition of human HDL in ways that could potentially improve their anti-inflammatory properties. The possibility that this may have occurred in the present study cannot be ruled out and warrants further investigation.

In conclusion, we have shown that niacin improves endothelial dysfunction and inhibits vascular inflammatory responses by increasing the vascular redox state and scavenging ROS without significantly affecting lipid levels. These findings indicate that niacin has multiple potential cardioprotective properties. They also offer a new insight into its mechanism of action in vivo.

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Disclosures
None.

References
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Evidence that niacin inhibits acute vascular inflammation and improves endothelial dysfunction independent of changes in plasma lipids

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**Materials and methods**

**Animal studies**

Three groups of male New Zealand White (NZW) rabbits (n=6-9/group) weighing approximately 2.2 kg (Institute of Medical and Veterinary Science, South Australia) received either regular chow (n=9) or chow supplemented with 0.6% (n=9) or 1.2% (n=6) (wt/wt) niacin (Sigma-Aldrich) for 14 days before and for 24 h after inserting a non-occlusive silastic collar around the left common carotid artery under general anaesthesia using isofluorane (4-5% for induction, 1.5-2% for maintenance)\(^1\). The animals were euthanized with an overdose of sodium pentobarbital (100 mg/kg i.v) 24 h after collar insertion. The collared segment of the left common carotid artery and the corresponding segment of the non-collared, right common carotid artery, as well as the thoracic aorta were excised, placed in ice-cold sterile saline, and cleaned of fat and connective tissue. All the procedures were approved by the Sydney South West Area Health Service Animal Welfare Committee.

**Immunohistochemistry**

A central ~3 mm section from each collared and non-collared carotid artery was fixed in 4% (v/v) cold paraformaldehyde, stored in 70% (v/v) ethanol, embedded in paraffin and sectioned (5 µm) for immunohistochemical analysis. For each animal, three 5 µm sections were chosen at 100 µm intervals and incubated at 37 °C for 1 hr with mouse anti-rabbit CD18 (1:200, AbD Serotec, Raleigh, NC), mouse anti-rabbit VCAM-1 (1:400), mouse anti-rabbit ICAM-1 (1:200) (both gifts from Dr M.Cybulsky, University of Toronto) monoclonal antibodies, or incubated at 4 °C overnight with mouse anti-human monoclonal antibodies against CD31 (1:50 dilution, DAKO), goat anti-human polyclonal antibodies against MCP-1 (1:200, Santa Cruz) or a rabbit anti-human polyclonal MPO antibody (1:200, DAKO) mixed with a biotin-conjugated polyclonal goat anti-rabbit antibody (1:200, DAKO) and rabbit serum (1:200, DAKO)\(^2\). Staining was visualized using the Horse Radish Peroxidase (HRP)-
3,3’ Diaminobenzidine (DAB) system (Envision Mouse Kit, DAKO), followed by counter staining with haematoxylin. The sections were imaged using an upright light microscope (Zeiss, Jena, Germany) at 5x or 10x magnification. DAB staining was quantified with ImageJ software (http://rsb.info.nih.gov/ij/). The polygon tool was used to quantify the total intima/media cross-sectional area and lumen circumference. The threshold for positive staining was defined by an independent observer who was blinded to the treatment. Positively stained areas were quantified by de-convolution. To account for variations in carotid artery size, the number of pixels representing endothelial VCAM-1, ICAM-1 and MCP-1 positive staining was divided by the circumference of the lumen. The resulting values were expressed as image units. CD18- and MPO-positive staining was expressed as a percentage of the total intima/media cross-sectional area. Endothelial integrity was assessed as the percentage of the endothelium staining positive for CD31. Antibody specificity was verified by staining three sections, 100µm apart, with appropriate IgG isotype controls. (Supplemental Fig. V).

**Assessment of endothelial function ex vivo**

For *ex vivo* assessment of endothelial function, isometric tension experiments were carried out on collared and non-collared carotid artery segments and thoracic aortic rings (~3 mm in length) within 4 h of sacrifice. The rings were placed in cold Krebs buffer solution (Sigma-Aldrich) that had been aerated with a gas mixture (95% O₂/5% CO₂). Ring viability was confirmed by incremental constriction in response to phenylephrine (Sigma-Aldrich) (2.0 g load for carotid artery segments and 2.5 g load for thoracic aortic rings). After preconstriction to 50% maximal response, the rings were exposed to incremental doses (0.001 to 10 µmol/L) of acetylcholine (Sigma-Aldrich) to assess endothelium-
dependent vasodilation, and then to sodium nitroprusside (Sigma-Aldrich) to assess endothelium-independent vasodilation.

The effect of hypochlorous acid (HOCl) on endothelial function in thoracic aortic rings isolated from animals that received normal chow or chow supplemented with 0.6% niacin was assessed by adding HOCl (Sigma-Aldrich) (final concentration 200 µmol/L) or vehicle (PBS) to the Krebs buffer solution, then incubating the rings at 37 °C for 10 min. Endothelium-dependent vasodilation was then assessed after removing the HOCl from the rings by washing (x3) with Krebs buffer solution.

For cGMP determinations, collared and non-collared carotid artery segments, as well as thoracic aortic rings (~3 mm in length) were incubated for 15 min at 37 °C in a 5% CO₂ incubator in Krebs buffer solution supplemented with 200 µmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich) to inhibit phosphodiesterases. The rings were then exposed to 1 µmol/L acetylcholine for 1 min at 37 °C, snap-frozen, and stored at -80 °C. Trichloroacetic acid 5% (w/v) was added to the frozen samples, which were homogenized as described and then acetylated with 4% (v/v) acetic anhydride in 0.64 mol/L KOH solution. The cGMP content of the samples was determined using an enzyme immunoassay kit according to the manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI).

**Effect of niacin on endothelial function and inflammation in vitro**

Thoracic aortic rings (~3 mm in length) isolated from animals that had received normal chow or chow supplemented with 0.6% niacin were incubated at 37 °C for 24 h in a 5% CO₂ incubator with human endothelial cell growth medium-serum free culture medium (EGM-SFM) (Cambrex Bio Science Walkersville, Bayonne, NJ) supplemented with 0.5 or 1 mmol/L niacin or vehicle (PBS). The rings were then used for endothelial function assessment, or incubated at 37 °C for a further 6 h with vehicle (PBS) or with TNF-α (10
ng/ml) (R&D System Inc., MN) to elicit an inflammatory response which was assessed by measuring changes in VCAM-1, ICAM-1 and MCP-1 mRNA levels by real-time PCR.

**Real-time PCR**

Total RNA was extracted by incubating carotid artery segments at 4 °C for 24 h in RNALater solution (Ambion, Austin, TX), then stored at -80 °C until use. Total RNA was isolated with TRIzol (Invitrogen) from RNALater-treated frozen tissues. The RNA was normalized to a concentration of 100 ng/µL using the SYBR Green II assay (Molecular Probes, Invitrogen, Carlsbad, CA) and reverse transcribed using iSCRIPT/iQ SYBR Green Supermix in a BioRad iQ5 thermocycler. Relative changes in mRNA levels were determined by the ΔΔCT method, using β-actin and 18S levels as controls. Primer pair sequences are included in Supplemental Table II.

**Determination of carotid artery and thoracic aorta total glutathione and protein content**

The total glutathione (GSH) content of frozen tissues was determined using the Cayman GSH assay kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s instructions. The method is based on a chemical reaction between the sulfhydryl group of GSH and 5,5’-dithio-bis-2-(nitrobenzoic acid) to produce 5-thio-2-nitrobenzoic acid. Protein concentrations were measured using the BCA-Protein Assay (Thermo Scientific, Rockford, IL).

**Biochemical analyses**

Blood was collected into EDTA-Na2 vacutainer tubes (Becton Dickinson) at the commencement of the study and prior to euthanasia of the animals. Plasma was isolated by centrifugation (1,000 g at 4 °C for 10 min), and then stored at -80 °C until used. Plasma total cholesterol4, triglyceride5 and non-esterified fatty acid6 levels were determined enzymatically. Plasma HDL cholesterol (HDL-C) levels were determined after precipitating apoB-containing lipoproteins with polyethylene glycol 60007. ApoA-I concentrations were determined immnoturbidimetrically using a sheep anti-rabbit apoA-I polyclonal antibody8.
All compositional analyses were performed on a Roche Diagnostics/Hitachi 902 AutoAnalyzer (Roche Diagnostics GmBH, Mannheim Germany). Plasma niacin levels were determined at the DMPK Bioanalytical Laboratory, Merck & Co., Inc (Rahway, NJ). Neutrophil counts were determined as described\(^9\). Briefly, total blood leukocytes were manually counted using a hemocytometer and a differential leukocyte determination was carried out on a blood smear treated with Diff Quik Stain (Lab. Aids, Australia).

**Statistics**

Data are expressed as mean ± SEM. One-way ANOVA and the Newman-Keul’s test were used to evaluate differences between groups. Acetylcholine and sodium nitroprusside dose response curves were compared between groups by one-way ANOVA for repeated-measures with Bonferroni corrections. The correlation coefficient of the association between CD18- and MPO-positive staining in the cross-sectional area of the intima/media was calculated by nonparametric correlation. All statistics were carried out using GraphPad Prism software version 4.03 (GraphPad Software, Inc. San Diego, CA). A value of p<0.05 was considered significant.
**Supplemental Table I.** Plasma lipid and apolipoprotein A-I levels at baseline and at sacrifice of animals treated with 0.6% or 1.2% niacin (wt/wt).

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<th>Baseline</th>
<th>Time of sacrifice</th>
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<tr>
<td></td>
<td>Control</td>
<td>0.6% niacin</td>
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<td>TC* (mmol/L)</td>
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<tr>
<td>ApoA-I (mg/mL)</td>
<td>1.01±0.05</td>
<td>1.02±0.10</td>
</tr>
</tbody>
</table>

*TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; NEFA, non-esterified fatty acid, apoA-I, apolipoprotein A-I.

Results are expressed as mean ± SEM, n=6. No significant differences were found between the groups, p>0.10 for all.
### Supplemental Table II. PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1 F</td>
<td>TAA AAT GCC TGG GAA GAT GG</td>
</tr>
<tr>
<td>VCAM-1 R</td>
<td>AAG AGA ATG TTG GGG ATG C</td>
</tr>
<tr>
<td>ICAM-1 F</td>
<td>TGC TCC GCC TTC CAC CAG</td>
</tr>
<tr>
<td>ICAM-1 R</td>
<td>TGG CAC CAC GCA GTC CTC</td>
</tr>
<tr>
<td>MCP-1 F</td>
<td>TCC ACA ACC CAA GAA CAC A</td>
</tr>
<tr>
<td>MCP-1 R</td>
<td>TCA CAG AGG GAA AGC ACA TA</td>
</tr>
<tr>
<td>β-actin F</td>
<td>GAT CGC TGA CCG TAT G</td>
</tr>
<tr>
<td>β-actin R</td>
<td>GTC GTA CTC CTG CTT GGT G</td>
</tr>
<tr>
<td>18S F</td>
<td>CGG CTA CCA CAT CCA AGG AA</td>
</tr>
<tr>
<td>18S R</td>
<td>GCT GGA ATT ACC GCG GCT</td>
</tr>
</tbody>
</table>
Supplemental Figure I. Endothelial integrity of non-collared and collared carotid arteries. Normocholesterolemic NZW rabbits were fed regular chow (Ctrl) or chow supplemented with 0.6% or 1.2% niacin (wt/wt) (n=6/group) for 2 weeks prior to implantation of a non-occlusive silastic collar around the left carotid artery. The animals were sacrificed 24 h post-collar insertion. Panel A shows representative pictures of sections from non-collared and collared arteries immunostained for CD31. Panel B shows the percentage of the endothelial circumference staining positive for CD31 in the non-collared (open bar) and collared (closed bars) arteries. Quantitation was carried out as described in the Materials and Methods. Data are expressed as mean ± SEM, n=6.
Supplemental Figure II. Blood neutrophil content. Carotid collars were inserted into normocholesterolemic NZW rabbits that were fed regular chow (Control) or chow supplemented with 0.6% or 1.2% niacin (wt/wt) (n=6/group) as described in the legend to supplemental Fig.1. Blood was collected at the time of euthanasia. Neutrophil counts were determined as described in Materials and Methods. Data are expressed as mean ± SEM, n=6.
Supplemental Figure III. Effect of incubation in medium on VCAM-1, ICAM-1 and MCP-1 mRNA levels. Segments of thoracic aortas from three animals that received normal chow were incubated in EGM-SFM culture medium at 37 °C for 30 h in a 5% CO₂ incubator. VCAM-1 (Panel A), ICAM-1 (Panel B) and MCP-1 (Panel C) mRNA levels were measured before (0 h, open bars) and after (30 h, closed bars) of incubation. Each experiment was carried out in triplicate. Data are expressed as mean ± SEM, n=3, p>0.05 for all.
Supplemental Figure IV. Plasma niacin levels. Plasma niacin concentrations were determined for each of six rabbits supplemented with 0.6 (Panel A) or 1.2% niacin (Panel B). Niacin levels were measured at days 2, 7 and 14 after commencement of supplementation.
Supplemental Figure V. IgG isotype control for immunostaining. Representative pictures of sections of collared arteries from normocholesterolemic NZW rabbits (n=6) fed regular chow. Sections were immunostained for relative IgG isotype control of mouse anti-rabbit VCAM-1 (Panel A), mouse anti-rabbit ICAM-1 (Panel B), goat anti-human MCP-1 (Panel C), mouse anti-rabbit CD18 (Panel D), rabbit-anti-human MPO (Panel E) and mouse anti-human CD31 (Panel F).
References


