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Niacin Inhibits Vascular Inflammation via the Induction of Heme Oxygenase-1

Ben J. Wu, PhD; Kang Chen, BMedSci; Philip J. Barter, MD, PhD; Kerry-Anne Rye, PhD

Background—Heme oxygenase-1 (HO-1) is a cytoprotective protein whose expression is consistently associated with therapeutic benefits in a number of pathological conditions such as atherosclerotic vascular disease and inflammation. Niacin is a pleiotropic drug that slows the progression of coronary artery disease and increases serum levels of the HO-1 enzymatic product bilirubin. This study asks if the cardioprotective properties of niacin involve the induction of HO-1.

Methods and Results—New Zealand White rabbits received chow or chow supplemented with 0.6% (wt/wt) niacin for 2 weeks. Acute vascular inflammation was induced in the animals by placing a nonocclusive silastic collar around the left common carotid artery. At 24 hours after collar implantation, serum bilirubin and vascular, liver, and spleen HO-1 messenger RNA levels were significantly increased. Vascular inflammation was decreased in the niacin-supplemented animals compared with control. Treatment of the animals with tin protoporphyrin-IX, a global HO inhibitor, or HO-1 small interfering RNA to knock down carotid artery HO-1 attenuated the ability of niacin to inhibit vascular inflammation. Treatment of cultured human coronary artery endothelial cells with niacin increased HO-1 expression by activating the nuclear factor-E2–related factor 2/p38 mitogen-activated protein kinase signaling pathway and inhibiting tumor necrosis factor α–induced endothelial inflammation. The antiinflammatory effects of niacin in human coronary artery endothelial cells were mimicked by bilirubin and abolished by incubation with tin protoporphyrin-IX and knock down of nuclear factor-E2–related factor 2.

Conclusions—Niacin activates HO-1 in vivo and in vitro. Induction of HO-1 may be partly responsible for the vascular protective properties of niacin. (Circulation. 2012;125:150-158.)

Key Words: cardiovascular diseases • drugs • endothelium • inflammation

Niacin is a broad-spectrum lipid-modifying agent that reduces plasma triglyceride, low-density lipoprotein, and lipoprotein(a) levels while raising that of high-density lipoproteins. Niacin also reduces plasma free fatty acid levels, albeit transiently. Niacin has been shown in several human clinical trials to promote regression of atherosclerosis. However, subsequent to the recent termination of the Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health (AIM-HIGH) trial because of a lack of effect on clinical cardiovascular events, it is apparent that more definitive evidence of the cardiovascular benefit of niacin will have to await the results of the ongoing Heart Protection Study 2 Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) study that has randomized >25 000 subjects.

Clinical Perspective on p 158

In contrast to the large body of knowledge on the mechanisms by which statins protect against cardiovascular disease, the cardioprotective mechanism(s) of niacin are not understood. Such mechanistic studies have the capacity to identify potential new cardioprotective targets.

In recent years, evidence has accumulated from in vitro and in vivo studies that at least some of the potentially cardioprotective properties of niacin are independent of changes in plasma lipid levels. For example, niacin inhibits inflammation, oxidative stress and monocyte adhesion in cultured human aortic endothelial cells. It also promotes angiogenesis in cultured mouse brain endothelial cells.

More recently we have reported that niacin attenuates acute vascular inflammation and improves endothelial dysfunction in normolipidemic, nonatherosclerotic New Zealand White (NZW) rabbits independent of changes in plasma lipid levels. This suggests that, in addition to promoting beneficial changes in plasma lipids, niacin may prevent cardiovascular disease by inhibiting vascular inflammation via mechanisms that are independent of its lipid-modifying effects. We report here that 1 possible mechanism by which this may occur is the ability of niacin to induce heme oxygenase-1 (HO-1).
Heme oxygenases catalyze the oxidative cleavage of heme into equimolar amounts of carbon monoxide, iron, and biliverdin, which is converted to bilirubin by biliverdin reductase. Humans express 2 isoforms of HO: HO-1, an inducible isoform, and HO-2, which is constitutively expressed. There is mounting evidence that pharmacological up-regulation of HO-1 protects against atherosclerosis, inflammation, and oxidation. Induction of HO-1 activity in animals, including adenoviral-mediated transfer of the HO-1 gene, markedly reduces atherosclerosis. Moreover, HO-1 gene delivery inhibits neointima formation after vascular injury. Expression of HO-1 is also protective in animal models of cardiac ischemia/reperfusion injury and pulmonary hypertension and in cardiac transplantation arteriosclerosis. Inhibition of HO-1, by contrast, exacerbates atherosclerosis. Heme oxygenase-1–deficient mice also develop chronic inflammation.

It is likely that the cardioprotective properties of HO-1 are mediated by products of HO-1 metabolism rather than by HO-1 itself. For example, bilirubin has antioxidant and anti-inflammatory properties, and its plasma concentration correlates inversely with cardiovascular risk. Carbon monoxide confers protection in several animal models of cardiovascular injury and disease, and the iron that is produced by HO-1 generates endothelial progenitor cells from bone marrow.

Intravenous and long-term oral administration of niacin elevates serum bilirubin levels by complex mechanisms that include increased splenic and hepatic HO-1 activity.

Given that bilirubin has known cardioprotective effects, we hypothesized that its formation downstream of HO-1 induction by niacin may explain some of the lipid-independent cardioprotective properties of the drug.

**Methods**

**Animal Studies**

Four groups of male NZW rabbits (n=6/group) weighing ~2.2 kg (Institute of Medical and Veterinary Science, South Australia) were maintained on regular chow (Groups 1 and 2) or chow supplemented with 0.6% (wt/wt) niacin (Sigma-Aldrich) (Groups 3 and 4). The animals received daily intraperitoneal (ip) injections of saline (Groups 1 and 3) or the HO pharmacological inhibitor Sn-protoporphyrin IX (SnPp) (Frontier Scientific, Logan, UT) (7.5 mg/kg body weight) (Groups 2 and 4) for 14 days before and for 24 hours after inserting a nonocclusive silastic collar around the left common carotid artery under general anesthesia.

Heme oxygenase-1 was knocked down with small interfering RNA (siRNA) in an additional 4 groups of rabbits (n=6/group). These animals received regular chow (Groups 5 and 6) or chow supplemented with 0.6% (wt/wt) niacin (Groups 7 and 8) for 2 weeks before implantation of a nonocclusive silastic collar around the carotid artery. Saline (200 μL) containing 40 μg of HO-1 siRNA (sense and antisense: 5′-CAAGGAGAACCCGGUCUACTT-3′ and 5′-GUAGACCGGGUUCUCCUUUGT-3′, respectively) and 10 μL FuGENE 6 (Roche Diagnostics GmbH, Mannheim Germany) was loaded into the space between the collar and carotid artery at the time of collar implantation to knock down local vascular HO-1 in the Group 5 and 7 animals. Saline (200 μL) containing scrambled siRNA (sense and antisense: 5′-CGUUCUUAACUCACAGUGAGGAAA-3′ and 5′-UCCUCUACCUGAGUGUAAAGC-3′, respectively) was loaded into the space between the collar and carotid artery at the time of collar implantation in the control animals (Groups 6 and 8).

The animals were euthanized with an overdose of sodium pentobarbital (100 mg/kg iv) 24 hours after collar insertion. The collared section of the left common carotid artery and the corresponding section of the noncollared right common carotid artery, as well as the liver and spleen, were placed in ice-cold saline and cleaned of fat and connective tissue. All procedures were approved by the Sydney Local Health Network Animal Welfare Committee.

**Immunohistochemistry**

A central ~3-mm section from each collared and noncollared 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. The sections were incubated at 37°C for 1 hour with mouse antiarabbit CD18 (1:200) (AbD Serotec, Raleigh, NC), mouse antiarabbit vascular cell adhesion molecule (VCAM)-1 (1:400), or mouse antiarabbit intercellular adhesion molecule (ICAM)-1 (1:200) (both gifts from Dr Myron I. Cybulsky, MD, PhD, University of Toronto, Canada) monoclonal antibodies. Staining was visualized using the Horse Radish Peroxidase-3,3′-Diaminobenzidine system (Envision Mouse Kit, DAKO, Glostrup, Denmark), followed by counter staining with hematoxylin. The sections were imaged using an upright light microscope (Zeiss, Jena, Germany) at 5× or 10× magnification. Diaminobenzidine 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 deconvolution. To account for variations in carotid artery size, the number of pixels representing endothelial VCAM-1– and ICAM-1–positive staining was divided by the circumference of the lumen.

**Bilirubin Determination and HO Activity Assay**

Serum was isolated from the euthanized animals by centrifugation (2000g at 4°C for 10 minutes), mixed with cold methanol (1:4, v/v), then centrifuged (2000g at 4°C for 10 minutes). Bilirubin levels in the supernatant were quantified using an LC18 column connected to a Shimadzu high-performance liquid chromatography system. Bilirubin was eluted with a linear gradient from 100% solvent A (methanol/100 mM ammonium acetate, pH 5.1; 3.2, v/v) to 100% solvent B (methanol) over 18 minutes. Bilirubin was detected at 455 nm.

Heme oxygenase activity was determined in microsomes from rabbit tissues and human coronary artery endothelial cells (HCAECs) (Cell Applications, San Diego, CA). The tissues were frozen and pulverized in liquid nitrogen, resuspended in PBS with protease inhibitors (Sigma-Aldrich), then homogenized. The HCAECs were lysed (3 freeze-thaw cycles). The tissue homogenates and cell lysates were centrifuged at 15 000g at 4°C for 20 minutes. The supernatants were collected and ultracentrifuged at 100 000g at 4°C for 1 hour, and the resulting microsomal pellets were suspended in buffer A (250 mM/L sucrose, 20 mM/L Tris, pH 7.4). For determination of HO activity, microsomal protein (100–600 μg) was mixed with rat liver microsomes (200 μg), 1 mM/L NADPH, 2 mM/L D-glucose-6-phosphate, 1 U glucose-6-phosphate dehydrogenase, and 2.5 mM/L heme (1 μL, Sigma-Aldrich) in 25% dimethyl sulfoxide in 100 μL buffer A on ice, then incubated at 37°C in dark for 1 hour. The reaction was stopped by adding ethanol/dimethyl sulfoxide (95:5, v/v, 100 μL). The samples were centrifuged at 13 000g for 5 minutes and bilirubin (a measure of HO enzyme activity) was quantified in the resulting supernatants.

**Cell Culture**

Human coronary artery endothelial cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (Cell Applications) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO2 incubator. To silence nuclear
transcription factor NF-E2–related factor (Nrf2) expression, HCAECs were transfected at 37°C for 48 hours with 200 pmol of specific siRNA (SMARTpool [mixture of 4 different target-specific sequences], Thermo Scientific, Lafayette, CO) or a scrambled siRNA control (Invitrogen, Carlsbad, CA) using the PotiMEM/Oligofectamine system (Invitrogen, Carlsbad, CA). Knockdown of Nrf2 in total cell lysates (by 63±7.8%) was confirmed by Western blotting.

When the cells were confluent, the culture medium was replaced with serum-free Dulbecco’s modified Eagle’s medium, and the incubation continued for a further 12 hours. The HCAECs (2×10^5 cells/6-well plate) were then incubated at 37°C with 1 mmol/L niacin for 0, 1, 2, 4, 6, and 24 hours, with 0, 0.25, 0.5, and 1 mmol/L niacin for 6 hours, or without or with 1 mmol/L niacin in the absence or presence of 20 μmol/L SnPP for 6 hours. Heme oxygenase-1 and HO-2 levels and Nrf2 expression were quantified by Western blotting and real-time polymerase chain reaction. Heme oxygenase activity was determined as described above.

For the cell-signaling experiments, HCAECs (2×10^5 cells/6-well plate) were preincubated for 1 hour with PD98059, SB203580, SP600125, or LY294004 (Merck, Kilsyth, Australia) (final concentration 10 μmol/L), then incubated for a further 6 hours without or with 1 mmol/L niacin. Human coronary artery endothelial cells (2×10^5 cells/6-well plate) were also incubated in the presence of 1 mmol/L niacin for 0, 3, 5, 10, 20, and 30 minutes to determine p38 mitogen-activated protein kinase (MAPK) phosphorylation.

The ability of HO-1 to mediate the antiinflammatory effects of niacin were assessed by incubating HCAECs (2×10^5 cells/6-well plate) with 0, 0.5, and 1 mmol/L niacin without or with 20 μmol/L SnPP for 16 hours, with 0, 1, 5, and 10 μmol/L bilirubin or with 10 μmol/L tricarbonyl dichlororuthenium(II) dimers (RuCo) or 10 μmol/L ferrous sulfate (FeSO_4) (Sigma-Aldrich) for 2 hours. The HCAECs were then incubated for a further 6 hours without or with tumor necrosis factor (TNF)-α (1 ng/mL).

**Western Blotting**

Human coronary artery endothelial cells were washed with ice-cold PBS and lysed in 20 mmol/L Tris buffer (pH 7.5) containing 0.5 mmol/L EDTA-Na_2, 0.5 mmol/L EGTA-Na_2, and protease inhibitors. Cellular proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). The membranes were incubated overnight using rabbit polyclonal antibodies against HO-1 (1:500) (StressGen, Victoria, Canada), HO-2 (1:200) (Santa-Cruz Biotechnology, Santa Cruz, CA), 38 MAPK (1:1000), and phospho-p38 MAPK (1:500) (Cell Signaling, Danvers, MA), as well as an anti-α-actin mouse monoclonal antibody (1:3000) (Sigma-Aldrich). Nuclear proteins were isolated using a nuclear extraction kit (Pierce, Rockford, IL), and Western blotted with an antirabbit Nrf2 polyclonal antibody (1:500) (Santa-Cruz Biotechnology). Antirabbit or antimouse immunoglobulin G horse-radish peroxidase (Abcam, Cambridge, UK) were used as secondary antibodies. Immunoreactive proteins were detected by chemiluminescence and analyzed using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA).

**Real-Time Polymerase Chain Reaction**

Liver and spleen were incubated in RNAlater solution (Ambion, Austin, TX) at 4°C for 24 hours, then stored at −80°C until use. Total RNA was isolated from the RNAlater-treated frozen tissues using TRIzol (Invitrogen, Carlsbad, CA) and extracted from HCAECs using TRI reagent (Sigma-Aldrich). 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/Pq 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 shown in online-only Data Supplement Table I.

**Statistics**

Data are expressed as mean±SEM. The Wilcoxon-Mann-Whitney Rank Sum test was used to evaluate differences between groups. All statistics were carried out using KaleidaGraph software version 3.6 (Synergy Software, Reading, PA). A value of P<0.05 was considered significant.

**Results**

Niacin Protects Against Vascular Inflammation in Rabbits via the Induction of HO-1

Four groups of normocholesterolemic NZW rabbits (n=6/group) were used to determine whether niacin induces HO-1 and whether this induction mediates the lipid-independent antiinflammatory properties of niacin we reported previously. This was achieved by maintaining the rabbits on regular chow or chow supplemented with 0.6% (wt/wt) niacin and administering daily ip injections of saline or SnPP for 2 weeks preceding carotid collar placement. The niacin dose used in this study was ~1g/d (based on chow consumption of 150 g over 24 hours). This is equal to a midrange pharmacological dose that causes flushing in ~30% of patients. Relative to body weight, this dose is about 30 times higher in the rabbit than in humans; however, the bioavailability of niacin in the rabbit is lower than in humans. We have reported previously that plasma niacin levels are ~38 μmol/L for the animals supplemented with 0.6% niacin compared with 40 to 160 μmol/L in humans after daily oral ingestion of 1.5 g niacin. Adverse side effects were not observed in the rabbits that received chow supplemented with 0.6% niacin.

At 24 hours after collar implantation, HO-1 mRNA levels in the collared arteries increased 1.6±0.2–fold compared with the noncollared arteries in the control animals (P<0.05) (Figure 1A). Relative to the control animals, dietary supplementation with niacin increased noncollared and collared carotid artery HO-1 mRNA levels 2.2±0.4– and 1.9±0.3–fold, respectively (Figure 1A), liver HO-1 mRNA 3.8±0.8–fold (Figure 1B), and spleen HO-1 mRNA 5.1±1.4–fold (Figure 1B) (P<0.05 for all). Dietary supplementation with niacin also increased liver HO activity by 1.6±0.1–fold (Figure 1C), spleen HO activity 1.5±0.1–fold (Figure 1C), and serum bilirubin levels 2.2±0.4–fold (Figure 1D) (P<0.05 for all). Daily ip SnPP injections abolished these effects (Figure 1C and 1D).

As reported previously, the carotid collar increased endothelial expression of VCAM-1, ICAM-1, and neutrophil recruitment into the vessel wall. At 24 hours after collar implantation, endothelial VCAM-1 and ICAM-1 expression increased from 0.2±0.3 to 4.5±0.7 and 0.6±0.7 to 6.1±0.6 image units, respectively (P<0.01 for both). Neutrophil recruitment into the intima/media (CD18+ staining), increased from 0.5±0.4% in the noncollared arteries to 19.3±3.9% in the collared arteries (P<0.001 for all) (Figure 2A and 2B).

Relative to the control animals, dietary supplementation with 0.6% niacin decreased collar-induced endothelial expression of VCAM-1×60±12%, ICAM-1 expression by 68±10%, and neutrophil recruitment by 90±6% (P<0.05 for all) (Figure 2A and 2B). The ability of niacin to protect against collar-induced vascular inflammation was completely
abolished in the animals after global inhibition of HO-1 expression with daily ip SnPP injections (Figure 2A and 2B).

The antiinflammatory properties of niacin were also investigated by specifically knocking down HO-1 in carotid arteries by transfection with HO-1 siRNA.23 Four groups of rabbits (n = 6/group) received regular chow or chow supplemented with 0.6% (wt/wt) niacin for 2 weeks preceding carotid collar implantation, at which time HO-1 siRNA or scrambled siRNA (siControl) was loaded into the space between the collar and carotid artery. At 24 hours after collar implantation, induction of HO-1 activity by niacin in the noncollared and collared carotid arteries (A) and in the liver and spleen (B) are shown. C, Liver and spleen HO activity in animals that received regular chow or chow supplemented with 0.6% (wt/wt) niacin without (closed bars) and with (open bars) daily ip SnPP injections. D, Serum bilirubin levels in animals that received regular chow and chow supplemented with 0.6% (wt/wt) niacin without (closed bars) and with (open bars) daily ip SnPP injections. Data are expressed as mean±SEM, n = 6. *P<0.05 compared with control. #P<0.05 compared with noncollared artery, or non-SnPP. HO-1 indicates heme oxygenase-1; mRNA, messenger RNA; SnPP, Sn-protoporphyrin IX; and Ctrl, control.

Figure 1. Niacin induces HO-1 activity in NZW rabbits. Normocholesterolemic NZW rabbits were maintained for 2 weeks on a regular chow diet (Ctrl) or chow supplemented with 0.6% (wt/wt) niacin. During this time, the animals received daily ip injections of saline or SnPP. After 2 weeks, a perianterial carotid collar was inserted. The animals were euthanized 24 hours after collar insertion. Heme oxygenase-1 mRNA levels (fold difference relative to control) in the noncollared and collared carotid arteries (A) and in the liver and spleen (B) are shown. C, Liver and spleen HO activity in animals that received regular chow or chow supplemented with 0.6% (wt/wt) niacin without (closed bars) and with (open bars) daily ip SnPP injections. D, Serum bilirubin levels in animals that received regular chow and chow supplemented with 0.6% (wt/wt) niacin without (closed bars) and with (open bars) daily ip SnPP injections. Data are expressed as mean±SEM, n = 6. *P<0.05 compared with control. #P<0.05 compared with noncollared artery, or non-SnPP. HO-1 indicates heme oxygenase-1; mRNA, messenger RNA; SnPP, Sn-protoporphyrin IX; and Ctrl, control.

artery abolished the niacin-mediated protection against collar-induced endothelial expression of VCAM-1 (Figure 3D) and ICAM-1 (Figure 3E) and attenuated the niacin-mediated protection against neutrophil recruitment into the vessel wall by 29±7%, (Figure 3F) (P<0.05 for all).

To ascertain if the HO-1 siRNA also inhibited HO-1 activity, thoracic aortic segments from the control animals were transfected with HO-1 siRNA or scrambled siRNA (control) and incubated without or with niacin. Heme oxygenase-1 mRNA levels (online-only Data Supplement Figure 1A) and activity (online-only Data Supplement Figure 1B) were both decreased in the segments that were incubated with HO-1 siRNA (P<0.05 for both). Taken together, these results indicate that the lipid-independent antiinflammatory properties of niacin are mediated by the induction of HO-1.

Niacin Induces HO-1 Expression in HCAECs in a Time- and Concentration-Dependent Manner

To investigate the underlying mechanism of the induction of HO-1 by niacin that was observed in vivo (Figure 1), the above findings were replicated in vitro. Human coronary artery endothelial cells were incubated with 1 mmol/L niacin for 1 to 24 hours or with 0.25 to 1.0 mmol/L niacin for 6 hours. Cell lysates were then subjected to SDS-PAGE and Western blotted for HO-1. Relative to control, incubation with niacin for 1, 2, 4, 6, and 24 hours increased HO-1 protein levels 2.7±0.7–, 3.9±0.8–, 5.0±0.6–, 5.5±0.9–, and 4.5±1.0–fold, respectively, (Figure 4A) (P<0.05 for all).

When HCAECs were incubated for 6 hours with 0.25, 0.5, and 1 mmol/L niacin, HO-1 protein levels increased 1.3±0.3–, 2.6±0.8–, and 4.5±0.7–fold, respectively (Figure 4B) (P<0.05). The time- and concentration-dependent induction of HO-1 by niacin was confirmed by real-time polymerase chain reaction. Relative to control cells, incubation for 1, 2, 4, 6, and 24 hours with niacin increased HO-1 mRNA levels 2.1±0.3–, 2.4±0.4–, 2.8±0.3–, 3.3±0.7–, and 3.3±0.6–fold, respectively (Figure 4C) (P<0.05 for all). When the cells were incubated for 6 hours with 0.25, 0.5, and 1 mmol/L niacin, HO-1 mRNA levels increased 1.5±0.2–, 1.8±0.4–, and 3.4±0.8–fold (P<0.05), respectively (Figure 4D).

Incubation of HCAECs for 6 hours with 1 mmol/L niacin also increased HO activity by 1.7±0.2–fold (P<0.05) (online-only Data Supplement Figure II A). This effect was completely abolished when SnPP was included in the incubation. Incubation with niacin had no effect on HO-2 protein levels (online-only Data Supplement Figure II B).

Induction of HO-1 by Niacin Involves Activation of Nrf2 and the p38-MAPK Signaling Pathway

Heme oxygenase-1 mediates the dissociation of Nrf2 from the Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and translocates Nrf2 to the nucleus.26 The binding of Nrf2 to Keap1 is also regulated by extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (JNK), and p38 MAPK.27 To determine whether niacin mediates the nuclear translocation of Nrf2, HCAECs were incubated with 1 mmol/L niacin for 1 to 24 hours (Figure 5A). Western blotting of nuclear extracts showed that, relative to cells...
incubated without niacin, incubation with 1 mmol/L niacin for 1, 2, 4, 6, and 24 hours increased nuclear Nrf2 protein levels 1.8–H110060.8– (ns), 2.9–H110060.8– (ns), 3.4–H110061.2– (ns), 5.5–H110060.9– (P/H110210.05), and 5.1–H110060.9–fold (P/H110210.05), respectively (Figure 5A).

The effect of Nrf2 on the niacin-mediated induction of HO-1 was investigated by transfecting HCAECs with Nrf2 siRNA or siControl, then incubating with niacin. Western blotting of cell lysates showed that, compared with cells incubated without niacin, incubation with niacin increased HO-1 expression 12–H110062–fold, (P/H110210.05) in the cells transfected with scrambled siRNA (Figure 5B). When the cells were transfected with Nrf2 siRNA, the niacin-mediated increase in HO-1 expression was reduced by 82–H110069.5% (P/H110210.05) (Figure 5B), indicating that induction of HO-1 by niacin involves activation of Nrf2.

To investigate whether Erk, JNK, and p38 MAPK subfamilies and PI3K/Akt signaling pathways are also involved in the induction of HO-1 by niacin, HCAECs were preincubated with the Erk-, JNK-, and p38 MAPK–specific inhibitors PD98059, SP600125, and SB203580 and the PI3K/Akt–specific inhibitor LY294002, then incubated with niacin. Western blotting of cell lysates showed that, compared with the cells incubated without niacin, incubation with niacin alone increased HO-1 expression 4.3–H110060.7–fold, (P<0.05). When the HCAECs were preincubated with the p38 MAPK–specific inhibitor SB203580, the niacin-induced increase in HO-1 expression was reduced by 86±10% (P<0.05) (Figure 5C). In contrast, PD98059, a specific Erk inhibitor, SP600125, that specifically inhibits JNK, and the PI3K–specific inhibitor LY294002 did not affect niacin-mediated HO-1 induction (Figure 5C). When the cells were incubated for 3 to 30 minutes with 1 mmol/L niacin, p38 MAPK was rapidly phosphorylated. Relative to cells incubated without niacin, phosphorylated-p38 protein levels were increased 2.2–H110061.8– (ns), 5.6–H110063.2– (ns), 26.9±4.1– (P<0.05), 10.1±4.6– (ns), and 7.5±2.9–fold (ns), respectively, in cells incubated with 1 mmol/L niacin for 3, 5, 10, 20, and 30 minutes (Figure 5D). This suggests that p38 MAPK is involved in the niacin-induced increase of HO-1 expression in HCAECs.

Niacin Protects Against Cytokine-Induced Inflammation in HCAECs by Inducing HO-1 Activity

to determine whether the induction of HO-1 by niacin also protects against cytokine-induced inflammation in vitro, HCAECs were incubated with niacin and SnPP before TNF-α stimulation. Relative to what was observed for TNF-α–stimulated HCAECs incubated without niacin, incubation with 0.5 and 1 mmol/L niacin decreased VCAM-1 mRNA levels by 34±4 and 49±9%, respectively (P<0.05 for both) (Figure 6A, open bars). Intercellular adhesion molecule 1 mRNA levels were reduced by 36±6 and 58±9%, respectively (Figure 6A, closed bars) (P<0.05 for both). This antinflammatory effect of niacin was abolished when SnPP was included in the incubations (Figure 6A).
We have reported previously that niacin has anti-inflammatory properties that are independent of changes in plasma lipid levels in an animal model of acute vascular inflammation.7 The present in vivo and in vitro studies show that the mechanism of these anti-inflammatory properties involves the induction of HO-1 expression. We have further confirmed these results by showing that the anti-inflammatory effects of niacin are mimicked by the HO-1 metabolic product bilirubin and that induction of HO-1 by niacin involves nuclear inflammatory effects in Figure 6A and 6B. Human coronary artery endothelial cells were incubated with bilirubin preceding TNF-α stimulation. Incubation with TNF-α increased VCAM-1 (Figure 6C, open bars) and ICAM-1 (Figure 6C, closed bars) expression 9.4±0.5– and 9.3±0.7-fold, respectively, compared with control (P<0.05 for both). Preincubation with 1, 5, and 10 μmol/L bilirubin inhibited the TNF-α–mediated increase in VCAM-1 expression by 25±5.8%, 49±9.3%, and 68±5.2% respectively, and ICAM-1 expression by 25±4.7%, 49±9.0%, and 65±4.7%, respectively (Figure 6C) (P<0.05 for all). Incubation with the 2 other HO-1 enzymatic products CO and Fe2+ did not affect the TNF-α–mediated increase in VCAM-1 and ICAM-1 expression (Figure 6C). This suggests that the anti-inflammatory effects that were observed in the niacin-treated NZW rabbits (Figure 2) may have been mediated by increased plasma bilirubin levels (Figure 1D).

**Discussion**

To determine whether the antiinflammatory effect of niacin in TNF-α–activated HCAECs also involved HO-1 induction and Nrf2 activation, we transfected HCAECs with Nrf2 siRNA or siControl. When the scrambled siRNA-transfected cells were further incubated with niacin, the TNF-α–induced increase in VCAM-1 (Figure 6B, open bars) and ICAM-1 (Figure 6B, closed bars) mRNA levels was decreased by 43±8% and 47±9%, respectively (P<0.05 for both). Niacin, by contrast, did not reduce VCAM-1 and ICAM-1 mRNA levels in the Nrf2 siRNA-transfected cells (Figure 6B). This indicates that niacin induces HO-1 via activation of Nrf2, which then protects against cytokine-induced inflammation in HCAECs.

Given that niacin increases circulating levels of the HO-1 enzymatic product bilirubin in rabbits (Figure 1D), we next investigated whether bilirubin may have mediated the anti-inflammatory effects in Figure 6A and 6B. Human coronary artery endothelial cells were incubated with bilirubin preceding TNF-α stimulation. Incubation with TNF-α increased VCAM-1 (Figure 6C, open bars) and ICAM-1 (Figure 6C, closed bars) expression 9.4±0.5– and 9.3±0.7-fold, respectively, compared with control (P<0.05 for both). Preincubation with 1, 5, and 10 μmol/L bilirubin inhibited the TNF-α–mediated increase in VCAM-1 expression by 25±5.8%, 49±9.3%, and 68±5.2% respectively, and ICAM-1 expression by 25±4.7%, 49±9.0%, and 65±4.7%, respectively (Figure 6C) (P<0.05 for all). Incubation with the 2 other HO-1 enzymatic products CO and Fe2+ did not affect the TNF-α–mediated increase in VCAM-1 and ICAM-1 expression (Figure 6C). This suggests that the anti-inflammatory effects that were observed in the niacin-treated NZW rabbits (Figure 2) may have been mediated by increased plasma bilirubin levels (Figure 1D).
translocation of the transcription factor Nrf2 and activation of the p38-MAPK signaling pathway.

The induction of HO-1 activity, increased HO-1 expression, and increased serum bilirubin levels after administration of niacin have been reported in both animal and human studies. For example, HO-1 activity in liver and spleen is rapidly increased in rats after intraperitoneal injection of niacin.22,23 Dietary supplementation with niacin also increases hepatic HO-1 mRNA levels in rats.30 In humans, long-term oral administration of niacin at pharmacological doses increases serum bilirubin levels by ≈9%.20 Moreover, in a recent randomized multicenter controlled trial, administration of niacin elevated serum total bilirubin levels by 10% to 20% over 48 weeks.21 We extend these findings in the present study by showing that 2 weeks of dietary supplementation with niacin increases carotid artery, liver, and spleen HO-1 mRNA levels in NZW rabbits (Figure 1A and 1B). Relative to control animals receiving regular chow, niacin supplementation also increased liver (Figure 1C) and spleen HO activity (Figure 1C) and serum bilirubin levels (Figure 1D). These effects were abolished by cotreatment with SnPP (Figure 1C and 1D). These findings suggest that the niacin-mediated elevation in serum bilirubin levels reported in humans may, at least in part, be a consequence of the induction of HO-1 activity.
The results of the present study also indicate that the elevated serum bilirubin levels reported in humans treated with niacin may explain the lipid-independent antiinflammatory properties of the drug. Evidence for this comes from the experiments showing that (1) preincubation of HCAECs with bilirubin protects against TNF-α–mediated endothelial inflammation (Figure 6C) and (2) global inhibition of HO activity by SnPP abolishes the ability of niacin to elevate serum bilirubin levels (Figure 1D) and inhibits collar-induced acute vascular inflammation (Figure 2).

In contrast to the inhibition of the antiinflammatory effects of niacin in animals with global inhibition of HO by SnPP (Figure 2), specific inhibition of vascular HO-1 (Figure 3A) only partially abolished the ability of niacin to inhibit collar-induced acute vascular inflammation (Figure 3F). This is most likely due to low siRNA transduction in the collared arteries. Furthermore, whereas global inhibition of HO with SnPP essentially abolished the niacin-mediated increase in serum bilirubin levels (Figure 1D), these levels remained elevated in the niacin-treated animals with localized vascular inhibition of HO-1 (Figure 3C). This result was expected given that local HO-1 inhibition was confined to the collared carotid artery and the increased serum bilirubin levels reflect HO-1 induction by niacin in multiple organs, including the liver and spleen (Figure 1B). It should also be noted that siRNA could not be used to inhibit global induction of HO-1 and that, as SnPP inhibits HO induction as a complex with heme, it could not be used to inhibit HO in collared carotid arteries.

Heme oxygenase-1 is transcriptionally up-regulated by several stimuli, including heme, oxidative stress, signaling proteins, and organic chemicals. This is because the promoter region of the HO-1 gene contains multiple copies of the antioxidant response element that binds translocated Nrf2 and up-regulates HO-1 expression.8 Our data showing that niacin increases nuclear translocation of Nrf2 (Figure 5A) and that niacin-induced HO-1 expression is reduced by transfection with Nrf2-siRNA in cultured endothelial cells (Figure 5B) indicate that the niacin-dependent increase of HO-1 is mediated mainly through transcriptional regulation by Nrf2.

Multiple kinase signaling pathways are involved in the induction of HO-1.20 Mitogen-activated protein kinases, such as Erk, JNK, and p38 MAPK play key roles in Nrf2 activation and increase HO-1 expression in various cell types.31 In human endothelial cells, the polyphenolic compound fisetin induces HO-1 expression by activating p38 MAPK and Nrf2,32 whereas dimethyl sulfoxide induces HO-1 expression via JNK and Nrf2 but not the p38 MAPK or Erk pathways.33 Niacin also phosphorylates and activates p38 MAPK but not Erk or JNK in skeletal muscle.54 It also stimulates ERK phosphorylation in human embryonic kidney cells and epithelial carcinoma cells.56 In addition, nicotinamide, a major metabolite of niacin, stimulates ERK phosphorylation in human myeloblastic leukemia cells.57 In the present study, exposure of HCAECs to niacin rapidly phosphorylated p38 MAPK (Figure 5D) as has been reported for simvastatin,38 inflammatory cytokines such as interleukin-10,39 and other stimuli.40 The current results confirm this selectivity by showing that inhibition of p38 MAPK, but not ERK or JNK, prevents HO-1 induction by niacin in HCAECs (Figure 5C). The precise mechanism of the early phosphorylation of p38 MAPK that causes activation of HO-1 gene transcription by niacin in endothelial cells remains to be clarified.

In conclusion, we have shown that niacin inhibits vascular inflammatory responses by induction of HO-1 activity, nuclear translocation of Nrf2, and activation of the p38 MAPK pathway. These findings provide an insight into the mechanisms of the antiinflammatory properties of niacin and suggest that its ability to induce HO-1 may be useful for inhibiting the vascular inflammation which is responsible for much of the tissue damage that occurs as a consequence of acute coronary events.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Niacin reduces plasma triglyceride, low-density lipoprotein, and lipoprotein(a) levels while raising that of high-density lipoproteins. Niacin also reduces plasma free fatty acid levels, albeit transiently. Niacin has been shown to promote regression of atherosclerosis and reduce cardiovascular events in humans. Until recently, niacin has not been used widely because it causes severe flushing that most people consider unacceptable. Partial alleviation of these side effects by use of extended release formulations, as well as combined therapy with the prostaglandin receptor antagonist laropiprant has increased niacin use, especially in association with statins. Combination therapy with niacin and a statin reduces the residual risk that persists in many people with dyslipidemia who are already being treated with a high dose of statin. In contrast to the large body of knowledge about the mechanisms by which statins protect against cardiovascular disease, the cardioprotective effects of niacin are not understood. The authors have reported previously that niacin inhibits vascular inflammation independent of changes in plasma lipid levels. The work described in this report demonstrates that the lipid-independent inhibition of vascular inflammation by niacin involves the induction of heme oxygenase-1 via activation of nuclear factor-E2-related factor 2 and the p38 mitogen-activated protein kinase signaling pathway. Heme oxygenase-1 is a cytoprotective protein whose expression is associated with therapeutic benefits in several pathological conditions such as atherosclerosis and inflammation. These results indicate that some of the cardioprotective effects of niacin may be due to its ability to induce heme oxygenase-1.
SUPPLEMENTAL MATERIAL

Niacin Inhibits Vascular Inflammation via the Induction of Heme Oxygenase-1

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### Supplemental Table I. PCR primer sequences

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Supplemental Figure I. Effects of HO-1 siRNA on niacin-mediated induction of HO-1 mRNA levels and HO activity in the thoracic aorta

Thoracic aortic segments isolated from rabbits that had received normal chow were incubated at 37 °C in a 5% CO₂ incubator with endothelial cell growth medium-serum free culture medium (EGM-SFM). The segments were transfected by incubation at 37 °C for 48 h with 400 pmol of HO-1 siRNA (siHO-1, open bars) or scrambled siRNA (siControl, closed bars) as described in the Methods, then incubated for a further 6 h in the absence or presence of 1 mM niacin. HO-1 mRNA levels were quantified by real-time PCR and expressed as fold change relative to siControl (Panel A). HO activity is shown in Panel B. Data are expressed as mean±SEM of three independent experiments. *p<0.05 compared to cells incubated without niacin. #p<0.05 compared to siControl.
Supplemental Figure II. Niacin induces HO activity but does not affect HO-2 levels in HCAECs. HCAECs were incubated without or with 1 mM niacin for 6 h in the absence or presence of 20 µM SnPP prior to quantifying HO activity as described in the Methods (Panel A) (*p<0.05 compared to cells incubated without niacin. #p<0.05 compared to cells incubated without SnPP). HCAECs were also incubated in the absence or presence of 1 mM niacin for 6 h, lysed and subjected to SDS-PAGE and Western blotting with anti-HO-2 or anti-α-actin antibodies. Quantification of HO-2 relative to β-actin is shown in Panel B. All data represent the mean±SEM of three independent experiments.