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

Running title: Wu et al.; Anti-inflammatory properties of niacin

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Journal Subject Codes: [90] Lipid and lipoprotein metabolism; [95] Endothelium/vascular type/nitric oxide; [138] Cell signalling/signal transduction
Abstract:

**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 which 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 non-occlusive silastic collar around the left common carotid artery. At 24 h post-collar implantation, serum bilirubin, and vascular, liver and spleen HO-1 mRNA levels were significantly increased. Vascular inflammation was decreased in the niacin-supplemented animals compared with control. Treatment of the animals with tin protoporphyrin-IX (SnPP), a global HO inhibitor, or HO-1 siRNA to knock down carotid artery HO-1, attenuated the ability of niacin to inhibit vascular inflammation. Treatment of cultured human coronary artery endothelial cells (HCAECs) with niacin increased HO-1 expression by activating the nuclear factor-E2-related factor 2 (Nrf2)/p38 mitogen-activated protein kinase signaling pathway, and inhibiting tumor necrosis factor-α-induced endothelial inflammation. The anti-inflammatory effects of niacin in HCAECs were mimicked by bilirubin, and abolished by incubation with SnPP and knock down of Nrf2.

**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.

**Key words:** cardiovascular diseases; drugs; endothelium; inflammation
Niacin is a broad spectrum, lipid-modifying agent that reduces plasma triglyceride, low density lipoprotein and Lp(a) levels, while raising that of high density lipoproteins (HDL). Niacin also reduces plasma free fatty acid levels, albeit transiently\(^1\). Niacin has been shown in several human clinical trials to promote regression of atherosclerosis\(^2-4\). 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 more than 25,000 subjects.

In contrast to the large body of knowledge regarding 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\(^5\). It also promotes angiogenesis in cultured mouse brain endothelial cells\(^6\).

More recently we have reported that niacin attenuates acute vascular inflammation and improves endothelial dysfunction in normolipidemic, non-atherosclerotic New Zealand White (NZW) rabbits independent of changes in plasma lipid levels\(^7\). 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 one possible mechanism by which this may occur is the ability of niacin to induce heme oxygenase-1 (HO-1).

Heme oxygenases (HOs) catalyze the oxidative cleavage of heme into equimolar amounts of carbon monoxide (CO), iron, and biliverdin, which is converted to bilirubin by biliverdin reductase. Humans express two isoforms of heme oxygenase: 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, pulmonary hypertension, and in cardiac transplant arteriosclerosis. Inhibition of HO-1, by contrast, exacerbates atherosclerosis. HO-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 anti-oxidant and anti-inflammatory properties and its plasma concentration correlates inversely with cardiovascular risk. CO 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 approximately 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 intra-peritoneal (ip) injections of saline (Groups 1 and 3) or the HO pharmacological inhibitor, Sn-protoporphyrin IX (SnPP) (Frontier Scientific, Logan, UT) (7.5mg/kg body weight) (Groups 2 and 4) for 14 days before and for 24 h after inserting a non-occlusive silastic collar around the left common carotid artery under general anaesthesia.

HO-1 was knocked down with siRNA in an additional four 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 prior to implantation of a non-occlusive silastic collar around the carotid artery. Saline (200 μL) containing 40 μg of HO-1 siRNA (Sense and antisense: 5’-CAAGGAGAACCCTTCGUCUACTT-3’ and 5’-

GUAGACCCGGGUUCUCUUUGTT-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’-

CGUUCUAAACUCACAGGUGAGGGAA-3’ and 5’-

UUCCUCACCUGUGAGGUAAAGACG-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 h post-collar insertion. The collared section of the left common carotid artery and the corresponding section of the non-collared, right common carotid artery, as well as the liver and spleen, were placed in ice-cold sterile 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 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. The sections were incubated at 37 °C for 1 h with mouse anti-rabbit CD18 (1:200) (AbD Serotec, Raleigh, NC), mouse anti-rabbit vascular cell adhesion molecule (VCAM)-1 (1:400), or mouse anti-rabbit intercellular adhesion molecule (ICAM)-1 (1:200) (both gifts from Dr M. Cybulsky, University of Toronto) monoclonal antibodies. Staining was visualized using the Horse Radish Peroxidase (HRP)-3,3’ Diaminobenzidine (DAB) system (Envision Mouse Kit, DAKO, Glostrup, Denmark), 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 that 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- and ICAM-1-positive staining was divided by the
circumference of the lumen. The resulting values were expressed as image units. CD18-positive staining was expressed as a percentage of the total intima/media cross-sectional area.

**Bilirubin determination and heme oxygenase activity assay**

Serum was isolated from the sacrificed animals by centrifugation (2,000xg at 4 °C for 10 min), mixed with cold methanol (1:4, v/v), then centrifuged (2,000xg at 4 °C for 10 min). Bilirubin levels in the supernatant were quantified using an LC18 column connected to a Shimadzu HPLC system\(^{15}\). Bilirubin was eluted with a linear gradient from 100% solvent A (methanol/100 mmol/L ammonium acetate, pH 5.1; 3:2, v/v) to 100% solvent B (methanol) over 18 minutes. Bilirubin was detected at 455 nm.

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

**Cell Culture**
HCAECs were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (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 h with 200 pmol of specific siRNA (SMARTpool (mixture of four different target-specific sequences, Thermo Scientific, Lafayette, CO) or a scrambled siRNA control (Invitrogen, Carlsbad, CA) using the PotiMEM/Oligfectamine 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 DMEM, and the incubation continued for a further 12 h. The HCAECs (2x10^5 cells/6-well plate) were then incubated at 37 °C with 1 mM niacin for 0, 1, 2, 4, 6 and 24 h, with 0, 0.25, 0.5 and 1 mM niacin for 6 h, or without or with 1 mM niacin in the absence or presence of 20 μM SnPP for 6 hours. HO-1 and HO-2 levels, and Nrf2 expression were quantified by Western blotting and real-time PCR. HO activity was determined as described above.

For the cell signalling experiments, HCAECs (2x10^5 cells/6-well plate) were pre-incubated for 1 h with PD98059, SB203580, SP600125, or LY294004 (Merck, Kilsyth, Australia) (final concentration 10 μM), then incubated for a further 6 h without or with 1 mM niacin. HCAECs (2x10^5 cells/6-well plate) were also incubated in the presence of 1 mM niacin for 0, 3, 5, 10, 20 and 30 min to determine p38 mitogen-activated protein kinase (MAPK) phosphorylation.

The ability of HO-1 to mediate the anti-inflammatory effects of niacin were assessed by incubating HCAECs (2x10^5 cells/6-well plate) with 0, 0.5 and 1 mM niacin without or with 20 μM SnPP for 16 hours, with 0, 1, 5 and 10 μM bilirubin, or with 10 μM tricarbonyl...
dichlororuthenium(II) dimer (RuCO) or 10 μM ferrous sulfate (FeSO₄) (Sigma-Aldrich) for 2 h. The HCAECs were then incubated for a further 6 h without or with tumor necrosis factor (TNF)-α (1 ng/mL).

**Western Blotting**

HCAECs were washed with ice cold PBS and lysed in 20 mM Tris buffer (pH 7.5) containing 0.5 mM EDTA-Na₂, 0.5 mM EGTA-Na₂ 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, BC 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 the NE-PER extraction kit (Pierce, Rockford, IL), and western blotted with an anti-rabbit Nrf2 polyclonal antibody (1:500) (Santa-Cruz Biotechnology). Anti-rabbit or anti-mouse IgG-HRP (Abcam, Cambridge, UK) were used as secondary antibodies. Immunoreactive proteins were detected by chemiluminescence and analysed using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA).

**Real-time PCR**

Liver and spleen were incubated in RNAlater solution (Ambion, Austin, TX) at 4 °C for 24 h, 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/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 shown in Supplemental Table 1.

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 anti-inflammatory properties of niacin we reported previously7. 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 prior to carotid collar placement. The niacin dose used in this study was approximately 1g/day (based on chow consumption of 150 g over 24 h). This is equal to a mid-range, pharmacological dose that causes flushing in approximately 30% of patients26, 27. 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 about 38 μmol/L for the animals supplemented with 0.6% niacin7, compared to 40-160 μmol/L in humans following daily oral ingestion of 1.5 g niacin28. Adverse side effects were not observed in the rabbits that received chow supplemented with 0.6% niacin.
At 24 h post-collar implantation, HO-1 mRNA levels in the collared arteries increased 1.6±0.2-fold compared to the non-collared arteries in the control animals (p<0.05) (Figure 1A). Relative to the control animals, dietary supplementation with niacin increased non-collared 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, 1D).

As reported previously, the carotid collar increased endothelial expression of VCAM-1, ICAM-1 and neutrophil recruitment into the vessel wall7. At 24 h post-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 non-collared arteries to 19.3±3.9% in the collared arteries (p<0.001 for all) (Figure 2A, 2B).

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

The anti-inflammatory properties of niacin were also investigated by specifically knocking down HO-1 in carotid arteries by transfection with HO-1 siRNA25. Four groups of rabbits (n=6/group) received regular chow or chow supplemented with 0.6% (wt/wt) niacin for 2
weeks prior to 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 h post-collar implantation, induction of HO-1 activity by niacin in the collared carotid arteries was not affected in the animals that received scrambled siRNA. When HO-1 siRNA was introduced into the space between the collar and the carotid artery, arterial HO-1 mRNA levels decreased by 85±19%, (Figure 3A) \( p<0.05 \) relative to scrambled siRNA). Local inhibition of carotid artery HO-1 siRNA did not affect niacin-mediated HO-1 induction in the liver (Figure 3B), or serum bilirubin levels (Figure 3C).

Relative to the control animals, dietary supplementation with niacin and placement of scrambled siRNA between the collar and the artery reduced collar-induced endothelial VCAM-1 expression by 51±12% (Figure 3D), ICAM-1 expression by 47±13% (Figure 3E), and neutrophil recruitment into the vessel wall by 91±6% (Fig. 3F) \( p<0.05 \) for all). Placement of HO-1 siRNA between the collar and the 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. HO-1 mRNA levels (Supplemental Figure 1A) and activity (Supplemental 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, anti-inflammatory 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. HCAECs were incubated with 1 mM niacin for 1-24 h, or with 0.25-1.0 mM niacin for 6 h. 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 h 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 h with 0.25, 0.5 and 1 mM niacin, HO-1 protein levels increased 1.3±0.3- (ns), 2.6±0.8- (ns) 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 PCR. Relative to control cells, incubation for 1, 2, 4, 6 and 24 h 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 (Fig. 4C) (p<0.05 for all). When the cells were incubated for 6 h with 0.25, 0.5 and 1 mM niacin, HO-1 mRNA levels increased 1.5±0.2- (ns), 1.8±0.4- (ns) and 3.4±0.8-fold (p<0.05) respectively (Figure 4D).

Incubation of HCAECs for 6 h with 1 mM niacin also increased HO activity by 1.7±0.2-fold (p<0.05) (Supplemental Figure 2A). This effect was completely abolished when SnPP was included in the incubation. Incubation with niacin had no effect on HO-2 protein levels (Supplemental Figure 2B).
Induction of HO-1 by niacin involves activation of Nrf2 and the p38-MAPK signalling pathway

HO-1 mediates the dissociation of Nrf2 from the Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, and translocates Nrf2 to the nucleus. The binding of Nrf2 to Keap1 is also regulated by extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). To determine whether niacin mediates the nuclear translocation of Nrf2, HCAECs were incubated with 1 mM niacin for 1-24 h (Figure 5A). Western blotting of nuclear extracts showed that, relative to cells incubated without niacin, incubation with 1 mM niacin for 1, 2, 4, 6 and 24 h increased nuclear Nrf2 protein levels 1.8±0.8- (ns), 2.9±0.8- (ns), 3.4±1.2- (ns), 5.5±0.9- (p<0.05), and 5.1±0.9-fold (p<0.05), respectively (Figure 5A).

The effect of Nrf2 on the niacin-mediated induction of HO-1 was investigated by transfecting HCAECs with Nrf2 siRNA, or scrambled siRNA (siControl), followed by incubation with niacin. Western blotting of cell lysates showed that, compared to cells incubated without niacin, incubation with niacin increased HO-1 expression 12±2-fold, (p<0.05) 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±9.5% (p<0.05) (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 pre-incubated 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 to the cells incubated without niacin, incubation with niacin alone
increased HO-1 expression 4.3±0.7-fold, (p<0.05). When the HCAECs were pre-incubated 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 3-30 min with 1mM niacin, p38 MAPK was rapidly phosphorylated. Relative to cells incubated without niacin, phosphorylated-p38 (p-p38) protein levels were increased 2.2±1.8- (ns), 5.6±3.2- (ns), 26.9±4.1- (p<0.05), 10.1±4.6- (ns), 7.5±2.9-fold (ns) respectively in cells incubated with 1 mM niacin for 3, 5, 10, 20 and 30 min (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 prior to TNF-α stimulation. Relative to what was observed for TNF-α-stimulated HCAECs incubated without niacin, incubation with 0.5 and 1 mM niacin decreased VCAM-1 mRNA levels by 34±4 and 49±9%, respectively (p<0.05 for both) (Figure 6A, open bars). ICAM-1 mRNA levels were reduced by 36±6 and 58±9%, respectively (Figure 6A, closed bars) (p<0.05 for both). This anti-inflammatory effect of niacin was abolished when SnPP was included in the incubations (Figure 6A).

To determine whether the anti-inflammatory effect of niacin in TNF-α-activated HCAECs also involved HO-1 induction and Nrf2 activation, HCAECs were transfected with
Nrf2 siRNA or scrambled siRNA (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 Figures 6A and 6B. HCAECs were incubated with bilirubin prior to 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 to control (p<0.05 for both). Pre-incubation with 1, 5 and 10 μM bilirubin inhibited the TNF-α-mediated increase in VCAM-1 expression by 25±5.8%, 49±9.3%, 68±5.2% respectively, and ICAM-1 expression by 25±4.7%, 49±9.0%, 65±4.7%, respectively (Figure 6C) (p<0.05 for all).

Incubation with the two 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

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. 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 translocation of the transcription factor Nrf2, and activation of the p38-MAPK signalling pathway.

The induction of HO-1 activity, increased HO-1 expression and increased serum bilirubin levels following administration of niacin has been reported in both animal and human studies. For example, HO-1 activity in liver and spleen is rapidly increased in rats following intraperitoneal injection of niacin. Dietary supplementation with niacin also increases hepatic HO-1 mRNA levels in rats. In humans, long-term oral administration of niacin at pharmacological doses increases serum bilirubin levels by about 9%. Moreover, in a recent Randomized Multicenter Controlled Trial, administration of niacin elevated serum total bilirubin levels by 10–20% over 48 weeks. We extend these findings in the present study by showing that two weeks of dietary supplementation with niacin increases carotid artery, liver and spleen HO-1 mRNA levels in NZW rabbits. Relative to control animals receiving regular chow, niacin supplementation also increased liver and spleen HO activity and serum bilirubin levels. These effects were abolished by co-treatment with SnPP. 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 anti-inflammatory properties of the drug. Evidence for this comes from the experiments showing that (i) pre-incubation of HCAECs with bilirubin protects against TNF-α-mediated endothelial inflammation.
(Figure 6C), and (ii) 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 anti-inflammatory 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, while 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 localised 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.

HO-1 is transcriptionally up-regulated by several stimuli, including heme, oxidative stress, signalling proteins, and organic chemicals. This is due to the promoter region of the HO-1 gene containing multiple copies of the antioxidant response element (ARE) that binds translocated Nrf2 and up-regulates HO-1 expression. Our data showing that niacin increases nuclear translocation of Nrf2 (Figure 5A), and 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 signalling pathways are involved in the induction of HO-1. MAPKs, such as Erk, JNK and p38 MAPK play key roles in Nrf2 activation and increase HO-1 expression in various cell types. In human endothelial cells, the polyphenolic compound fisetin induces HO-1 expression by activating p38 MAPK and Nrf2, whereas DMSO induces HO-1 expression via JNK and Nrf2, but not the p38 MAPK or ErK pathways. Niacin also phosphorylates and activates p38 MAPK, but not Erk or JNK in skeletal muscle. It also stimulates ERK phosphorylation in human embryonic kidney cells and epithelial carcinoma cells. In addition, nicotinamide, a major metabolite of niacin, stimulates ErK phosphorylation in human myeloblastic leukemia cells. In the present study, exposure of HCAECs to niacin rapidly phosphorylated p38 MAPK (Figure 5D), as has been reported for simvastatin, inflammatory cytokines such as interleukin-10 and other stimuli. 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 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 anti-inflammatory properties of niacin and suggest that its ability to induce HO-1 may be useful for inhibiting the vascular inflammation that is responsible for much of the tissue damage that occurs as a consequence of acute coronary events.

**Funding Sources:** This work was supported by the National Health and Medical Research Council of Australia (Grant 482800).
Conflict of Interest Disclosures: None

References:


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**Figure Legends:**

**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 periarterial carotid collar was inserted. The animals were sacrificed 24 h post-collar insertion. HO-1 mRNA levels (fold difference relative to control) in the non-collared and collared carotid arteries (**Panel A**), and in the liver and spleen (**Panel B**) are shown. **Panel C** shows 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. **Panel D** shows 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 to control. #p<0.05 compared to non-collared artery, or non-SnPP.
Figure 2. Global inhibition of HO activity prevents niacin from reducing acute vascular inflammation in NZW rabbit collared carotid arteries. Periarterial collars were inserted into NZW rabbits receiving regular chow (Control) or chow supplemented with 0.6% niacin (wt/wt) and daily ip injections of saline (closed bars) or SnPP (open bars) for 2 weeks as described in the legend to Figure 1. Representative pictures of sections from non-collared and collared arteries immunostained for VCAM-1, ICAM-1 and CD18 are shown in Panel A (bar=50 μm). Quantification of VCAM-1 and ICAM-1, and CD18 staining is shown in Panel B. Data are expressed as mean±SEM, n=6. *p<0.05 with versus without niacin.

Figure 3. Local inhibition of HO-1 activity prevents niacin from reducing acute vascular inflammation in NZW rabbit collared carotid arteries. NZW rabbits were maintained on regular chow or chow supplemented with 0.6% (wt/wt) niacin for 2 weeks prior to periarterial carotid collar insertion. HO-1 siRNA (siHO-1) (open circles) or scrambled siRNA (siControl) (closed circles) was loaded into the space between the collar and artery at the time of collar implantation. The animals were sacrificed 24 h post-collar insertion. HO-1 mRNA levels (fold difference relative to siControl) in the collared carotid arteries (Panel A) and the liver (Panel B) are shown. Serum bilirubin levels are shown in Panel C. Quantification of VCAM-1, ICAM-1 and CD18 positive staining in collared artery sections are shown in Panels D, E and F, respectively. Data are expressed as individual points with the cross-symbol indicating the mean ± SEM, n=6. *p<0.05 with versus without niacin. #p<0.05 siControl versus siHO-1

Figure 4. Niacin induces HO-1 expression in HCAECs. HCAECs were incubated with 1 mM niacin for 0-24 h (Panel A), or with 0-1 mM niacin for 6 h (Panel B). Cell lysates were
subjected to SDS-PAGE and Western blotting with anti-HO-1 or anti-α-actin antibodies as indicated. Results represent the intensity of HO-1 band relative to α-actin. Relative HO-1 mRNA levels (fold difference relative to control) in HCAECs are shown in Panels C and D, respectively. All data are expressed as the mean±SEM of three independent experiments. *p<0.05 compared to cells incubated without niacin.

Figure 5. Niacin induces HO-1 expression in HCAECs by activating Nrf2 and the p38-MAPK signalling pathway. HCAECs were incubated with 1 mM niacin for 0-24 h. Panel A shows Western blot analysis of Nrf2 and α-actin in nuclear extracts. Panel B shows Western blot analysis of HO-1 and α-actin in lysates of HCAECs transfected with scrambled siRNA (siControl, closed bars) or Nrf2 siRNA (open bars) for 48 h, then incubated in the absence or presence of 1 mM niacin for a further 6 h. Panel C shows Western blot analysis of HO-1 and α-actin in lysates of HCAECs pre-incubated with 10 μmol/L PD98059, SB203580, SP600125, or LY294002 for 1 h (open bars), then incubated for a further 6 h in the absence or presence of 1 mM niacin. Panel D shows Western blot analysis for phosphorylated p38-MAPK (p-p38) and p38-MAPK (p38) in the lysates of HCAECs incubated with 1 mM niacin for 0-30 min. Results are quantified as Nrf2 (Panel A) and HO-1 (Panels B and C) intensity relative to α-actin, and p-p38 relative to total p38 (Panel D). Data are expressed as the mean±SEM of three independent experiments. #p<0.05 siControl versus siNrf2, with versus without niacin. *p<0.05 compared to control.

Figure 6. Niacin and bilirubin protect against cytokine-induced inflammation in HCAECs via the induction of HO-1 activity. HCAECs were pre-incubated for 16 h with the indicated
concentrations of niacin in the absence or presence of SnPP (final concentration 20 μM), then stimulated for 6 h with TNF-α (1 ng/mL). **Panel A** shows VCAM-1 (open bars) and ICAM-1 (closed bars) mRNA levels. **Panel B** shows VCAM-1 (open bars) and ICAM-1 (closed bars) mRNA levels in HCAECs transfected with scrambled siRNA (siControl) or Nrf2 siRNA (siNrf2), incubated in the absence or presence of 1 mM niacin for a further 16 h, then stimulated for 6 h with TNF-α (1 ng/mL). **Panel C** shows VCAM-1 (open bars) and ICAM-1 (closed bars) expression in HCAECs pre-incubated for 2 h with bilirubin, RuCO, or FeSO₄, then stimulated for 6 h with TNF-α (1 ng/mL). Data are expressed as mean±SEM of three independent experiments. *p<0.05 compared to HCAECs stimulated with TNF-α in the absence of niacin or bilirubin.
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Circulation, published online November 17, 2011:
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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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.