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Niacin protects against abdominal aortic aneurysm formation via GPR109A independent mechanisms: role of NAD^+/nicotinamide

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Aims Chronic adventitial and medial infiltration of immune cells play an important role in the pathogenesis of abdominal aortic aneurysms (AAAs). Nicotinic acid (niacin) was shown to inhibit atherosclerosis by activating the anti-inflammatory G protein-coupled receptor GPR109A [also known as hydroxycarboxylic acid receptor 2 (HCA2)] expressed on immune cells, blunting immune activation and adventitial inflammatory cell infiltration. Here, we investigated the role of niacin and GPR109A in regulating AAA formation.

Methods and results Mice were supplemented with niacin or nicotinamide, and AAA was induced by angiotensin II (AngII) infusion or calcium chloride ($CaCl_2$) application. Niacin markedly reduced AAA formation in both AngII and $CaCl_2$ models, diminishing adventitial immune cell infiltration, concomitant inflammatory responses, and matrix degradation. Unexpectedly, GPR109A gene deletion did not abrogate the protective effects of niacin against AAA formation, suggesting GPR109A-independent mechanisms. Interestingly, nicotinamide, which does not activate GPR109A, also inhibited AAA formation and phenocopied the effects of niacin. Mechanistically, both niacin and nicotinamide supplementation increased nicotinamide adenine dinucleotide (NAD^+) levels and NAD^+-dependent Sirt1 activity, which were reduced in AAA tissues. Furthermore, pharmacological inhibition of Sirt1 abrogated the protective effect of nicotinamide against AAA formation.

Conclusion Niacin protects against AAA formation independent of GPR109A, most likely by serving as an NAD^+ precursor. Supplementation of NAD^+ using nicotinamide-related biomolecules may represent an effective and well-tolerated approach to preventing or treating AAA.

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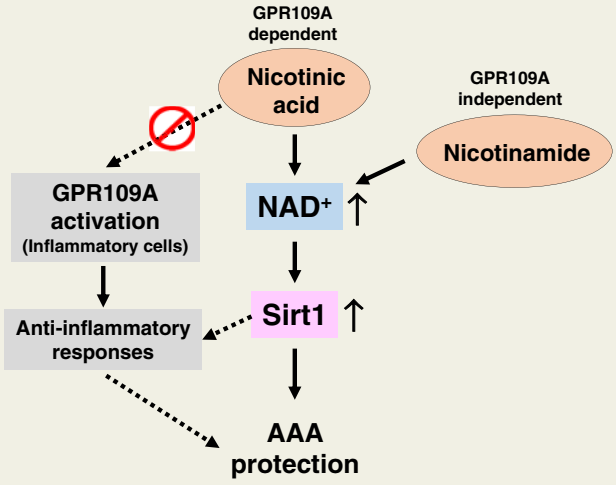
Graphical Abstract



Keywords Abdominal aortic aneurysm • Nicotinic acid • Nicotinamide • GPR109A • NAD+ • Sirt1

1. Introduction

Abdominal aortic aneurysms (AAAs) are characterized by localized structural deterioration of the aortic wall, leading to progressive aortic dilation and rupture.[1] Chronic adventitial and medial inflammation plays a key role in the pathogenesis of AAA through production of pro-inflammatory cytokines [i.e. monocyte chemoattractant protein 1 (MCP)-1, tumour necrosis factor (TNF)-α, and interleukin (IL)-6], reactive oxygen species, and matrix metalloproteinases (MMPs), which promote degradation of the vascular wall.[2] Targeting immune pathways can prevent the formation of experimental AAA,[3,4] suggesting a promising strategy for AAA treatment in humans. An ideal anti-inflammatory agent would not only be effective but safe, well-tolerated, and inexpensive for long-term use. In this regard, HMG Co-A reductase inhibitors (statins), which favourably modulate inflammation and oxidative stress, are safe and effective at reducing the complications of atherosclerosis.[5] While statins may be of modest benefit in AAA, they have not been proven to diminish the need for intervention, thereby exposing patients to complex invasive vascular procedures.[6]

Nicotinic acid (niacin) was the first hypolipidaemic agent shown to reduce mortality in humans[7] and remains in use as an alternative lipid-lowering agent in statin-intolerant patients, despite its poor tolerability. Niacin possesses numerous biological properties that suggest it may be highly efficacious in AAA, including elevation of high-density lipoprotein (HDL) cholesterol[8] and adiponectin levels,[9] inhibiting monocyte/macrophage inflammatory responses,[10,11] and modulating oxidative stress by supplying reducing equivalents, up-regulating heme oxidase-1,[12] and inhibiting nicotinamide adenine dinucleotide phosphate oxidase, which has been linked to the pathogenesis of AAA.[13,14] Additionally, niacin is an important precursor of nicotinamide adenine dinucleotide (NAD^+), a central metabolic cofactor that is required for the activity of multifunctional enzymes such as sirtuin-1,[15] deficiency of which in vascular smooth muscle cells was reported to promote aortic aneurysm.[16] However, to our knowledge, niacin has not been systematically studied in human or experimental models of AAA disease.

In a collar injury-induced atherosclerosis model, niacin was shown to inhibit adventitial vascular inflammatory cell infiltration and accumulation of myeloperoxidase (MPO),[17] which we recently reported to contribute to the pathogenesis of AAA.[18] The anti-atherosclerotic effects of niacin have been attributed to the activation of the G protein-coupled receptor GPR109A [also known as hydroxycarboxylic acid receptor 2 (HCA2)] expressed on immune cells, independent of its lipid-modifying effects.[19] GPR109A is highly expressed on adipocytes and inflammatory cells,[20] including neutrophils, which are critical to the pathogenesis of AAA.[21] GPR109A activation promotes neutrophil apoptosis and inhibits MPO release, thereby suppressing oxidative stress.[22] Thus, we hypothesized that GPR109A activation by niacin might also inhibit AAA formation by reducing vascular inflammation and matrix degradation.

Here, we investigated the role of niacin in preventing AAA using two different murine models: angiotensin II (AngII) infusion in low-density lipoprotein receptor (LDLR) knockout ($KO^{-/-}$) mice and calcium chloride ($CaCl_2$) application in wild-type (WT) mice. Niacin effectively inhibited AAA formation, but surprisingly, these effects were independent of GPR109A. Nicotinamide, which does not activate GPR109A, phenocopied the effects of niacin, which were mechanistically linked to restoration of aortic NAD^+ levels and Sirt1 activity. These results suggest that elevating NAD^+ levels and concomitant Sirt1 activity by nicotinamide-related biomolecules, which are safe and well-tolerated clinically, is a promising therapeutic strategy for AAA treatment and prevention.

2. Methods

2.1 Animals

Animal experimental protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Georgia at

Augusta University and complied with National Institute of Health guidelines. LDLR KO mice were purchased from Jackson Laboratory (Bar Harbor, ME). GPR109A KO mice were obtained from Dr Stefan Offermanns (Max Planck Institute for Heart and Lung Research, Germany). GPR109A KO mice were identified by PCR-based genotyping, and PCR confirmed that GPR109A gene expression was undetectable in whole bone marrow cells isolated from GPR109A KO mice (Supplementary material online, *Figure S1*).[20] Mice were anaesthetized by isoflurane vaporizer (0.5–1.0 L/min for oxygen flowmeter, 4–5% for induction, and 1–3% for maintenance, EZ Anesthesia Systems) and buprenorphine SR (1.2 mg/kg body weight) was administered subcutaneously prior to making the incision to relieve additional pain. Mice were euthanized with intraperitoneal pentobarbital 150 mg/kg, inhaled anaesthesia (isoflurane, 5%) followed by carbon dioxide (CO_2) narcosis followed by cervical dislocation or bilateral thoracotomy, in accordance with AVMA Panel 2007 recommendations and institutional IACUC guidelines.

2.2 AngII-induced AAA model

AngII (Enzo Life Sciences, 1000 ng/kg/min) was infused into male LDLR KO mice via osmotic mini-pumps (ALZET Model 2004) as described.[20] Mice were treated with oral niacin (0.3% wt/vol) or nicotinamide (0.1% or 0.4% wt/vol, purchased from Sigma-Aldrich)[8,19,23,24] supplemented in drinking water for 2 days prior to mini-pump implantation and continued throughout the study. In some experiments, mice were co-treated with 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX-527, 20 mg/kg body weight) to inhibit Sirt1 activity.[25] Blood pressure measurements were obtained using a validated tail-cuff method (Coda 6, Kent Scientific).[18] Serum cholesterol and triglyceride were quantified using commercial assays (Wako Pure Chemical Industries). Two to four weeks after mini-pump implantation, mice were euthanized, aortic outer diameter was measured, and tissues were collected for analysis.

2.3 $CaCl_2$-induced AAA model

Male C57Bl/6 mice received water with or without nicotinic acid (0.3% wt/vol) or nicotinamide (0.1% or 0.4% wt/vol) for 2 days prior to $CaCl_2$ induction of AAA using 0.5 mol/L of $CaCl_2$ (Sigma-Aldrich), as described previously.[18] After 3 weeks, animals were anaesthetized, aortic outer diameter was measured, and tissues were collected for analysis.

2.4 Gelatin zymography

Zymography to detect matrix metalloproteinase (MMP) activity was performed as previously described.[18] Briefly, protein lysate (600 μg) was placed in a non-reducing zymogram buffer and applied without boiling to a 10% zymogram gel (Bio Rad). Gels were incubated in 2% Triton X 100 at room temperature for 30 min, and then rinsed in distilled water for 5 min. Gels were incubated overnight at 37°C with gentle agitation and proteins were stained with Coomassie Brilliant Blue R-250 solution (Bio Rad) and de-stained with a solution containing 40% methanol, 10% acetic acid, and 50% water.

2.5 Immunohistochemistry

Paraffin-embedded cross sections (5 μm) were stained with haematoxylin and eosin (H&E), Verhoeff-van Gieson (VVG), or Alizarin red (Ricca Chemical Company), MPO (Abcam), Mac-3 (BD Pharmingen), MCP-1 (Novus Biologicals), and matrix metalloproteinase (MMP)-2/MMP-9 (Calbiochem) antibodies were used with the HistoMouse-SP kit (Invitrogen) or DAB Substrate kit (Vector Labs). The number of elastin breaks was counted in three sections per mouse to quantify elastin degradation.[26] Vascular calcification was detected by Alizarin Red staining. To quantify the immunostaining data, total stained area in the media and adventitial region was analysed using Image-Pro Plus Software.[27]

2.6 Quantitative PCR

Total RNA was extracted from aortic tissues with QIAzol Lysis Reagent, and purified with RNeasy Lipid Tissue Mini Kit (Qiagen). Real-time quantification of mRNA levels of the genes of interest was performed using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) per manufacturer's instructions. Normalized Ct values were subjected to statistical analysis and fold difference was calculated by ΔΔCt method as described previously.[28] Primers used in this study are listed in Supplementary material online, *Table S1*.

2.7 Measurement of respiratory burst in neutrophilic cells

Respiratory burst assay was performed as previously described.[29] In brief, human promyelocytic NB4 cells were differentiated into neutrophilic cells by treating with all-trans retinoic acid ATRA (1 μM, Sigma-Aldrich) for 5 days. Cells (5×10^4/well in 96 well plates) were incubated in phenol-free medium containing L-012 for 10 min, and phorbol 12-myristate 13-acetate (PMA, 100 nM) was applied in the presence or absence of niacin (50 μM or 300 μM). Luminescence was quantified using a POLARstar luminometer (BMG Labtech).

2.8 Measurement of vascular smooth muscle cell (VSMC) viability

Primary aortic VSMCs were grown to confluence, and serum-starved for 24 h, followed by pretreatment with niacin (10 μM) for 6 h. Then, cells were exposed to hydrogen peroxide (H_2O_2, 100 μM) and cell viability was assessed by trypan blue exclusion method. In brief, cells were rinsed with PBS after H_2O_2 treatment, and a mixture of 0.4% trypan blue and fresh culture media (1:5 ratio) was added. Cell viability in three randomly selected areas was evaluated by counting the number of dead cells per total number of cells.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Serum levels of MCP-1 were determined by Mouse CCL2/JE/MCP-1 DuoSet ELISA kit per manufacturer's instructions (R&D Systems).

2.10 Measurement of tissue levels of NAD^+ and Sirt1 activity

Aortic NAD^+ levels were determined using NAD/NADH Quantitation Colorimetric Kit (BioVision), and Sirt1 activity was assayed using Sirt1 Activity Assay Kit (Abcam) per manufacturer's instructions.

2.11 Western blotting

Protein extraction and western blotting were performed as described previously.[20] Sirt1 (Cell Signaling Technology) and GAPDH (Ambion) antibodies were used.

2.12 Statistical analysis

All statistical analysis was performed using GraphPad Software (GraphPad Software, Inc., USA). Results are expressed as mean ± SEM unless otherwise noted. Differences between two groups were analysed by Student's *t*-test. Multiple group datasets were evaluated for normality, and differences were analysed by one-way ANOVA followed by

Bonferroni *post hoc* analysis. *P*-values <0.05 were considered to be significant.

3. Results

3.1 Niacin protected against AngII-induced AAA formation

To investigate whether niacin protects against AAA formation, LDLR KO mice were treated with niacin (0.3% in drinking water) during AngII infusion for 2 weeks. This dose equates to approximately 2.5 g/day in a 70 kg person given the 10-fold greater metabolism of niacin in mice vs. humans.[8,19] Niacin supplementation decreased AAA incidence (*Figure 1A*), aortic diameter (*Figure 1B*) and plasma MPO levels (*Figure 1C*). Aortic MPO accumulation, macrophage infiltration, and MCP-1 expression were also reduced by niacin treatment (*Figure 1D*, Supplementary material online, *Figure S2*). Furthermore, niacin prevented elastin degradation (VVG staining in *Figure 1D* and *E*), in conjunction with reduced aortic MMP-2 and MMP-9 immunostaining (*Figure 1D*, *Supplementary material online*, *Figure S2*) and activity (zymography, *Figure 1F*). MMP-2/-9 and MCP-1 mRNA expression also showed a trend towards reduction, while TNFα was significantly decreased, in aortic tissues of niacin-treated mice (*Figure 1G*). *In vitro*, niacin inhibited the respiratory burst produced by neutrophilic NB4 cells (Supplementary material online, *Figure S3*) and enhanced VSMC viability following treatment with 100 μM H_2O_2 (Supplementary material online, *Figure S4*). In contrast, niacin did not alter systolic blood pressure in AngII-infused mice (Supplementary material online, *Figure S5*), nor did it significantly reduce plasma cholesterol or triglyceride levels during the 2-week course of this study (Supplementary material online, *Figure S6*).

3.2 Niacin treatment reduced $CaCl_2$-induced AAA formation

We next tested the effects of niacin using the $CaCl_2$-induced AAA model, which exhibits similar pathological characteristics found in human AAA, including calcification, vascular smooth muscle cell apoptosis, marked adventitial inflammatory cell infiltration, and more severe elastin degradation as compared to the AngII-induced AAA model.[30] Moreover, unlike the AngII model, the $CaCl_2$ model employs normolipidaemic, non-atherosclerotic mice, which eliminates potentially confounding systemic influences of niacin therapy. Niacin efficiently prevented $CaCl_2$-induced AAA formation (*Figure 2A*), in association with diminished elastin degradation (H&E and VVG staining), aortic macrophage infiltration and MMP-2/-9 expression (*Figure 2B* and Supplementary material online, *Figure S2*). Thus, niacin effectively prevented AAA formation in both animal models of AAA.

3.3 GPR109A gene deletion did not abrogate the beneficial effects of niacin against AAA formation

GPR109A activation in immune cells was reported to mediate the anti-atherosclerotic effects of niacin.[19] To investigate whether the protective effects of niacin against AAA formation are also mediated by GPR109A, GPR109A KO mice were treated with niacin, and AAA was induced by $CaCl_2$ application. Unexpectedly, GPR109A gene deletion did not impact the efficacy of niacin to protect against AAA formation (*Figure 3A*). Similarly, GPR109A deficiency did not alter niacin's ability to blunt aortic macrophage infiltration (Mac-3), MPO accumulation, or MCP-1 expression (*Figure 3B*, *Supplementary material online*, *Figure S2*). We also assayed MCP-1 levels in plasma of WT and GPR109A KO mice. Interestingly, there was a trend towards increased plasma MCP-1 levels in GPR109A KO mice as compared to WT mice in the absence of AAA induction (Sham, *Figure 3C*). AAA induction was associated with increased plasma MCP-1, and niacin treatment tended to reduce MCP-1 levels in both WT and GPR109A KO mice, although the data were not statistically significant. These results suggest that niacin exerts anti-inflammatory effects independent of GPR109A in the context of AAA.

3.4 Nicotinamide treatment protected against AngII-induced AAA formation

Since niacin's protective effects against AAA formation were found to be independent of GPR109A, we next tested nicotinamide, which exhibits similar metabolic effects as niacin but does not activate GPR109A on inflammatory cells,[31] and does not have lipid-lowering effects,[15] in the AngII infusion model. Interestingly, nicotinamide treatment (0.4%) also significantly reduced AAA formation (*Figure 4A*) and phenocopied the effects of niacin with regard to suppression of macrophage infiltration, elastin degradation, MPO accumulation and MCP-1 expression (*Figure 4B and C*, Supplementary material online, *Figure S2*). MMP-2 and -9 activities were also significantly reduced by nicotinamide treatment (zymography, *Figure 4D*). Like niacin, nicotinamide did not alter systolic blood pressure in AngII-infused mice (Supplementary material online, *Figure S5*). As expected, plasma lipid levels were not affected by nicotinamide treatment (Supplementary material online, *Figure S6*).

3.5 Nicotinamide treatment reduced $CaCl_2$-induced AAA formation

We next tested the effects of nicotinamide on AAA formation using the $CaCl_2$ application model. Nicotinamide (0.1% and 0.4%) was highly effective at inhibiting AAA formation induced by $CaCl_2$ application (*Figure 5A*), and these protective effects were accompanied by reduced MMP-2/-9 activities (*Figure 5B*), macrophage infiltration, and elastin degradation (*Figure 5C*, Supplementary material online, *Figure S2*). The $CaCl_2$ application model is also associated with aortic calcification, which is frequently observed in human AAAs.[30] Thus, we performed Alizarin red staining to quantify calcification,[27] which was found to be significantly reduced by nicotinamide treatment (*Figure 5D*). Taken together, these results demonstrate that nicotinamide, like niacin, strongly protected against AAA formation in both animal models, further suggesting a GPR109A-independent mechanism of action.

3.6 Niacin and nicotinamide restored aortic NAD^+ levels and Sirt1 activity

Since both niacin and nicotinamide function as NAD^+ precursors, we next examined whether NAD^+ levels are altered during AAA formation in the absence or presence of treatment with niacin or nicotinamide. Intriguingly, NAD^+ levels were markedly reduced in both AngII- and $CaCl_2$-induced AAA tissues and similarly restored by either niacin or nicotinamide treatment (*Figure 6A and B*). Sirt1 is a key NAD^+ consuming enzyme that regulates cellular metabolism,[32] and deletion of Sirt1 in vascular smooth muscle cells augmented aortic aneurysm formation.[16] Thus, we examined whether Sirt1 activity is regulated by niacin or nicotinamide in the context of AAA formation. Interestingly, we detected significantly reduced Sirt1 activity in aortic tissues in both experimental models that was similarly restored by either niacin or nicotinamide treatment (*Figure 6C and D*). Treatment with niacin or nicotinamide alone,

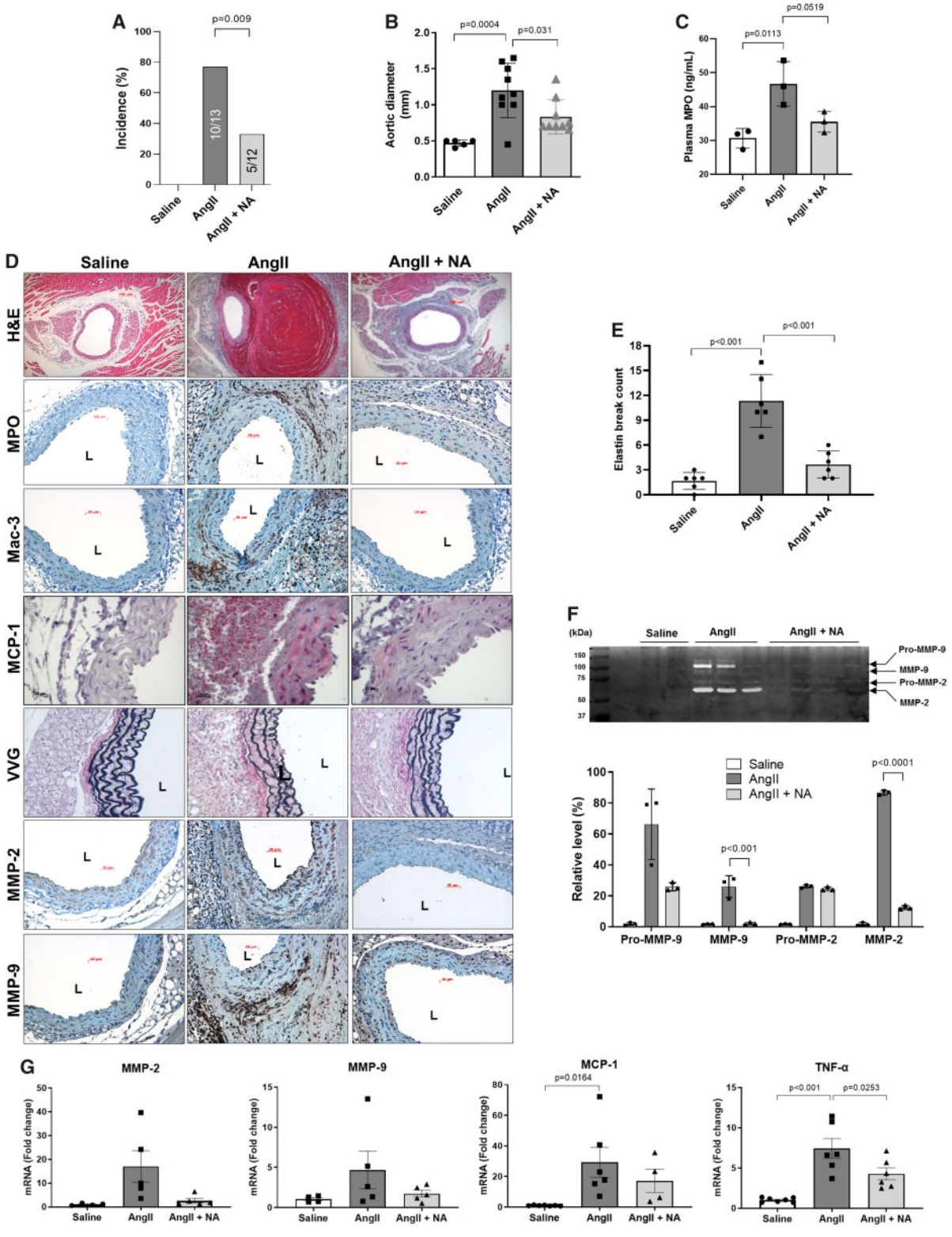


Figure 1 Niacin protected against AngII-induced AAA formation. AngII was infused via osmotic mini-pump in LDLR KO mice, which were treated with or without niacin in the drinking water. (*A*) AAA incidence ($n = 12–13$). (*B*) Aortic diameter ($n = 5–9$). (*C*) Plasma MPO levels (ELISA, $n = 3$). (*D*) Representative histology; H&E, MPO, Mac-3 (macrophages), VVG (elastin fragmentation), MMP-2/-9, and MCP-1. (*E*) Elastin break count ($n = 4–6$). (*F*) Representative zymogram (upper panel) and quantified data (bottom panel) for aortic MMP-2 and MMP-9 activities ($n = 3–4$). (*G*) mRNA expression of MMP-2 ($n = 4–5$), MMP-9 ($n = 4–5$), MCP-1 ($n = 4–7$), and TNFα ($n = 6–7$). Data were analysed using one-way ANOVA followed by Bonferroni *post hoc* analysis. AngII, angiotensin II; H&E, haematoxylin & eosin; L, lumen; Mac, macrophage; MCP-1, monocyte chemoattractant protein 1; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NA, nicotinic acid; TNFα, tumour necrosis factor α; VVG, Verhoeff-van Gieson.

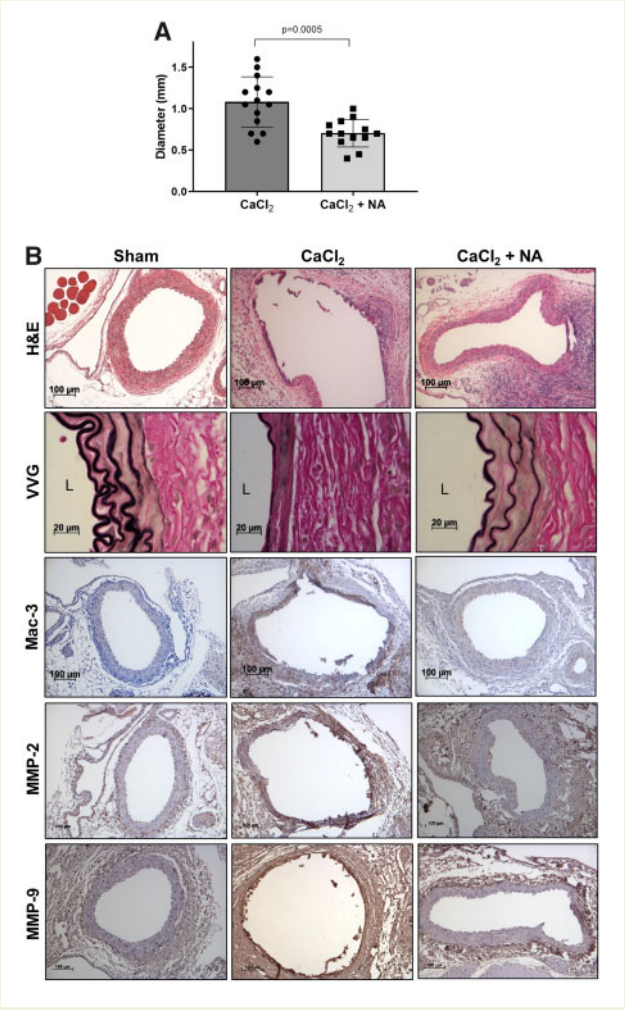


Figure 2 Niacin protected against $CaCl_2$-induced AAA formation. $CaCl_2$ was applied to the infrarenal aortae of C57Bl/6 mice. (*A*) AAA diameter ($n = 13–14$). (*B*) Representative histology; H&E, VVG (elastin fragmentation), Mac-3 (macrophages), and MMP-2/-9. Data were analysed using one-way ANOVA followed by Bonferroni *post hoc* analysis. $CaCl_2$, calcium chloride; H&E, haematoxylin & eosin; L, lumen; Mac, macrophage; NA, nicotinic acid; VVG, Verhoeff-van Gieson.

without inducing AAA formation, did not significantly increase NAD^+ levels or Sirt1 activity. In contrast, Sirt1 protein expression was not significantly altered in either the $CaCl_2$ or the AngII model in the absence or presence of niacin or nicotinamide treatment (*Figure 6E and F*, Supplementary material online, *Figure S7*). These results suggest that niacin/nicotinamide restore Sirt1 activity by primarily by repleting NAD^+ rather than by regulating Sirt1 protein expression.

3.7 Inhibition of Sirt1 activity abolished the protective effects of NAD^+ repletion on AAA formation

To investigate whether NAD^+ repletion is functionally dependent on Sirt1 activity, AngII-infused mice were treated with EX-527, a selective Sirt1 inhibitor. We first confirmed that EX-527 did not significantly influence the incidence of AngII-induced AAA in the absence of treatment with nicotinamide (AngII vs. AngII + EX-527, *Figure 7A*). Also, when administered along with a lower dose of AngII (500 ng/kg/min), EX-527 did not significantly increase the incidence of AAA (not shown) or aortic diameter (Supplementary material online, *Figure S8*). However, when co-administered to mice treated with 1000 ng/kg/min AngII, EX-527 blocked the ability of nicotinamide to prevent AAA formation (*Figure 7A and B*), elastin degradation, MPO accumulation and macrophage infiltration (*Figure 7D*, Supplementary material online, *Figure S2*). As expected, EX-527 prevented the increase in aortic Sirt1 activity in mice treated with nicotinamide (*Figure 7C*). Furthermore, MCP-1 and TNFα gene expression was significantly reduced by nicotinamide that was abrogated by co-treatment with EX-527 (*Figure 7E*), suggesting that the protective effects of nicotinamide against AAA formation are mediated by NAD^+ repletion/Sirt1 activation.

4. Discussion

Niacin is a lipid-lowering agent that also exhibits anti-inflammatory and anti-oxidative effects in many cell types. Using two distinct animal models of AAA, we report for the first time that niacin can effectively protect against AAA formation, in conjunction with reduced aortic immune cell infiltration, inflammation, and matrix degradation. Unexpectedly, these inhibitory effects of niacin were found to be independent of GPR109A signalling and shared by nicotinamide, which like niacin restored aortic NAD^+ levels and Sirt1 activity. These results suggest that nicotinamide and related biomolecules, which are safe, well-tolerated, and inexpensive, can potentially restore aortic NAD^+ levels and concomitant Sirt1 activity to protect against AAA formation.

4.1 Novel GPR109A-independent role of niacin-mediated AAA protection

GPR109A is a G protein-coupled receptor highly expressed on inflammatory cells (neutrophils and macrophages) that can be activated by various agonists, including niacin, to elicit anti-inflammatory effects. In neutrophils, GPR109A activation promotes apoptosis and inhibits release of MPO.[17] Deletion of GPR109A in bone marrow-derived cells prevented the ability of niacin to inhibit macrophage recruitment and atherosclerosis progression,[19] suggesting a critical role for this receptor in transducing niacin's vasculoprotective effects. We observed a trend toward increased expression of GPR109A mRNA in the aortas of AngII-infused mice (Supplementary material online, *Figure S9*), in conjunction with enhanced inflammatory cell infiltration (*Figures 1D and 4B*), which were reduced by niacin or nicotinamide treatment. However, deletion of GPR109A did not abrogate niacin's ability to prevent AAA or diminish aortic inflammation. These disparate findings with niacin in atherosclerosis versus AAA models are likely consistent with the unique pathophysiological features of these two vascular inflammatory disorders. Interestingly, Zhang *et al.*[33] reported that macrophage-specific Sirt1 deficiency enhanced MCP-1 expression and promoted AAA formation, suggesting that niacin-induced Sirt1 activation in macrophages could potentially contribute to the GPR109A-independent effects of niacin in preventing aneurysm formation. VSMC apoptosis, likely triggered in part by oxidative stress, is a major pathogenic feature of AAA.[3] Here, we demonstrated that niacin protected against H_2O_2-induced cytotoxicity in VSMCs, which do not express GPR109A (Supplementary material online, *Figure S4*). Watson *et al.*[16] also demonstrated that nicotinamide riboside, which likewise does not activate GPR109A, augmented VSMC survival, suggesting that the protective effects of niacin against AAA formation could be mediated in part by NAD^+ repletion in VSMC.

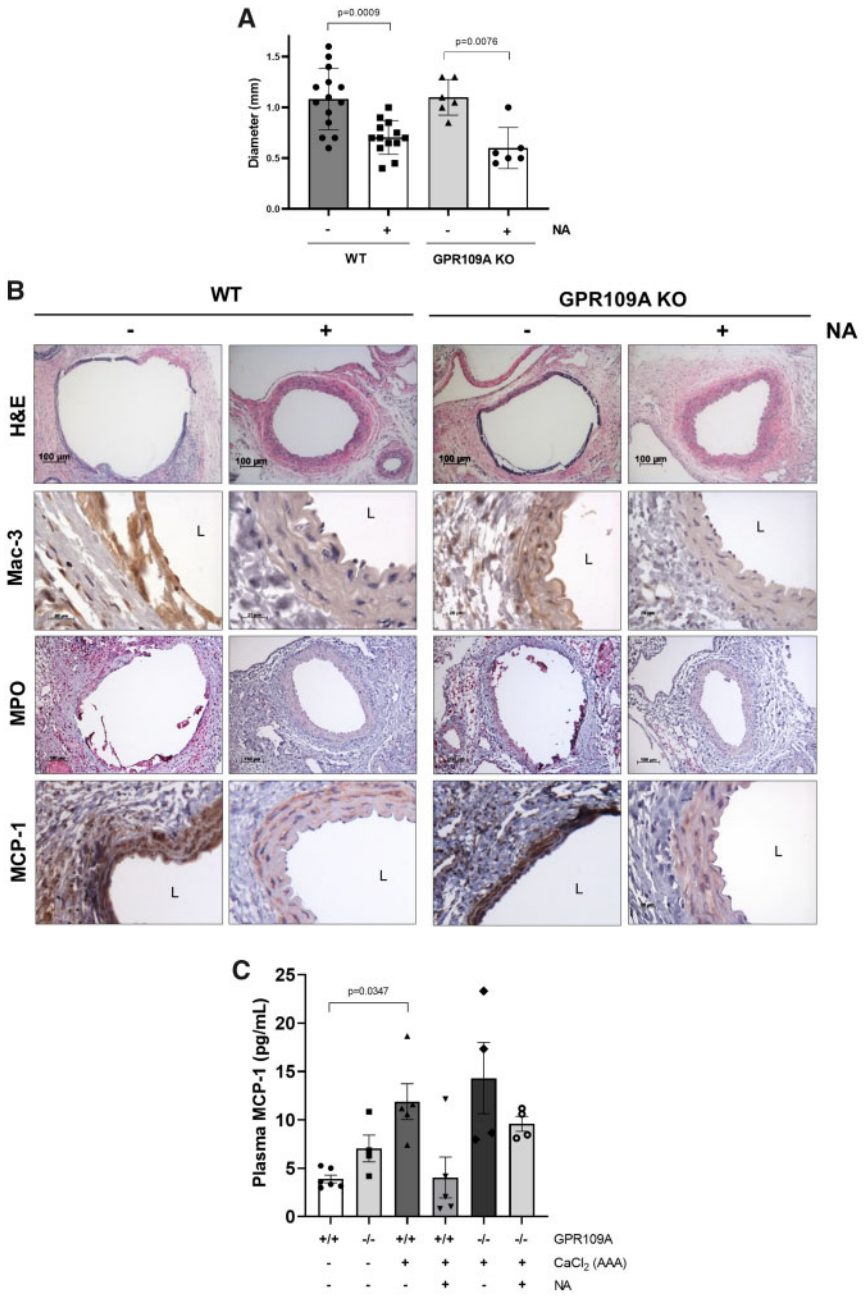


Figure 3 GPR109A gene deletion did not abrogate the protective effects of niacin against AAA formation in the $CaCl_2$ model. (*A*) AAA diameter ($n = 6$–15). (*B*) Representative histology; H&E, Mac-3 (macrophages), VVG (elastin fragmentation), MPO, and MCP-1. (*C*) Plasma MCP-1 levels (ELISA, $n = 4$–6). Data were analysed using one-way ANOVA followed by Bonferroni *post hoc* analysis. H&E, haematoxylin & eosin; L, lumen; Mac, macrophage; MCP-1, monocyte chemoattractant protein 1; MPO, myeloperoxidase; NA, nicotinic acid.

However, whether niacin acts specifically on VSMC, macrophages, and/or other cell types to prevent AAA formation remains to be definitively established.

4.2 Protective effects of NAD^+ repletion on AAA formation

Our data indicate that nicotinamide, which does not activate GPR109A, also inhibited AAA formation and phenocopied the effects of niacin, further supporting the hypothesis that GPR109A-independent mechanisms contribute to niacin's protective effects against AAA formation. These results led us to investigate NAD^+ metabolism in AAA since both niacin and nicotinamide are dietary precursors of NAD^+. NAD^+ levels are depleted with aging,[34] which is also an important risk factor for AAA formation. Although 16 mg of daily niacin is reportedly sufficient to meet the minimum requirement of NAD^+ synthesis in humans,[35] mounting evidence suggests that substantially greater NAD^+ levels could be beneficial to protect against

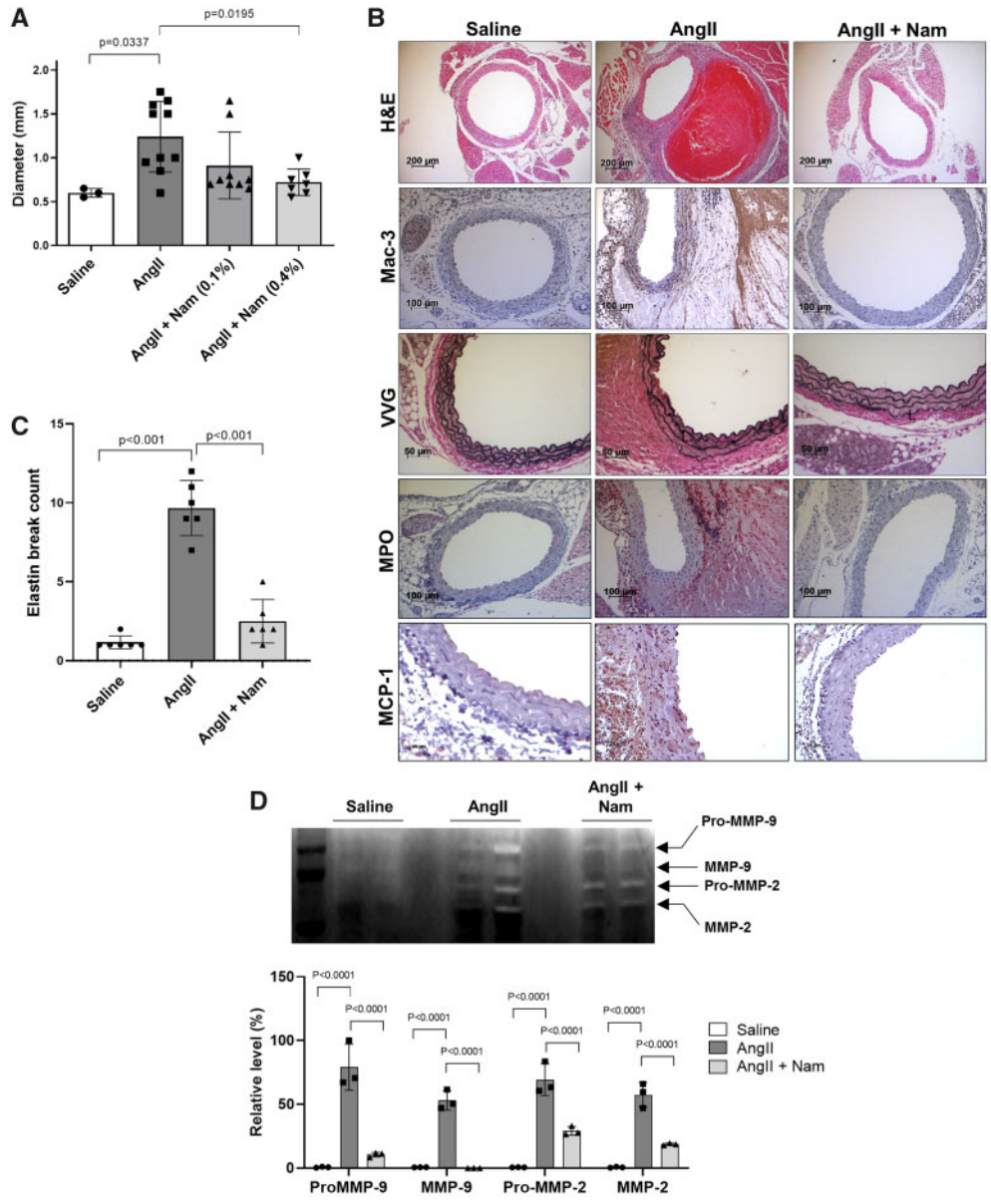


Figure 4 Nicotinamide reduced AngII-induced AAA formation. AngII was infused in LDLR KO mice via osmotic mini-pump, and mice were treated without or with nicotinamide in the drinking water. (*A*) AAA diameter ($n = 3$–10). (*B*) Representative histology; H&E, Mac-3 (macrophages), VVG (elastin fragmentation), MPO, and MCP-1. (*C*) Elastin break count ($n = 6$). (*D*) Representative zymogram (upper panel) and quantified data (bottom panel) for aortic MMP-2 and MMP-9 activities ($n = 3$). Data were analysed using one-way ANOVA followed by Bonferroni *post hoc* analysis. AngII, angiotensin II; H&E, haematoxylin & eosin; L, lumen; Mac, macrophage; MMP, matrix metalloproteinase; Nam, nicotinamide; VVG, Verhoeff-van Gieson.

cardiovascular disease.[15] NAD^+ is a substrate of NAD^+-consuming enzymes (i.e. sirtuins), and reduced NAD^+ levels and sirtuin activity with age are implicated in the pathogenesis of a variety of cardiovascular and metabolic diseases.[36] Moreover, expression of nicotinamide phosphoribosyltransferase (Nampt), an NAD^+ producing enzyme, in vascular smooth muscle cells was shown to protect against thoracic aortic aneurysm by maintaining genome integrity and preventing senescence in VSMCs,[16] further suggesting that NAD^+ metabolism is important to maintain vascular homeostasis and prevent aneurysm disease. In both AngII-infusion and $CaCl_2$ application models of AAA, we demonstrate reduced aortic NAD^+ levels, which were efficiently restored by either niacin or nicotinamide treatment, suggesting that NAD^+ depletion *per se* may contribute to the pathogenesis of AAA.

Dysfunctional perivascular adipose tissue (PVAT) regulates vascular pathobiology and may be involved in AAA formation. Visfatin, a PVAT-derived adipokine, was reported to promote VSMC proliferation by increasing nicotinamide nucleotide synthesis.[37] Furthermore, high-fat diet feeding in mice reduced the expression of Nampt, an enzyme that biosynthesizes NAD^+, in PVAT,[38] suggesting a mechanistic linkage between NAD^+ and PVAT-mediated vascular inflammation. In this study, we did not examine whether NAD^+ supplementation by niacin or nicotinamide specifically regulates PVAT function to modulate aneurysm formation.

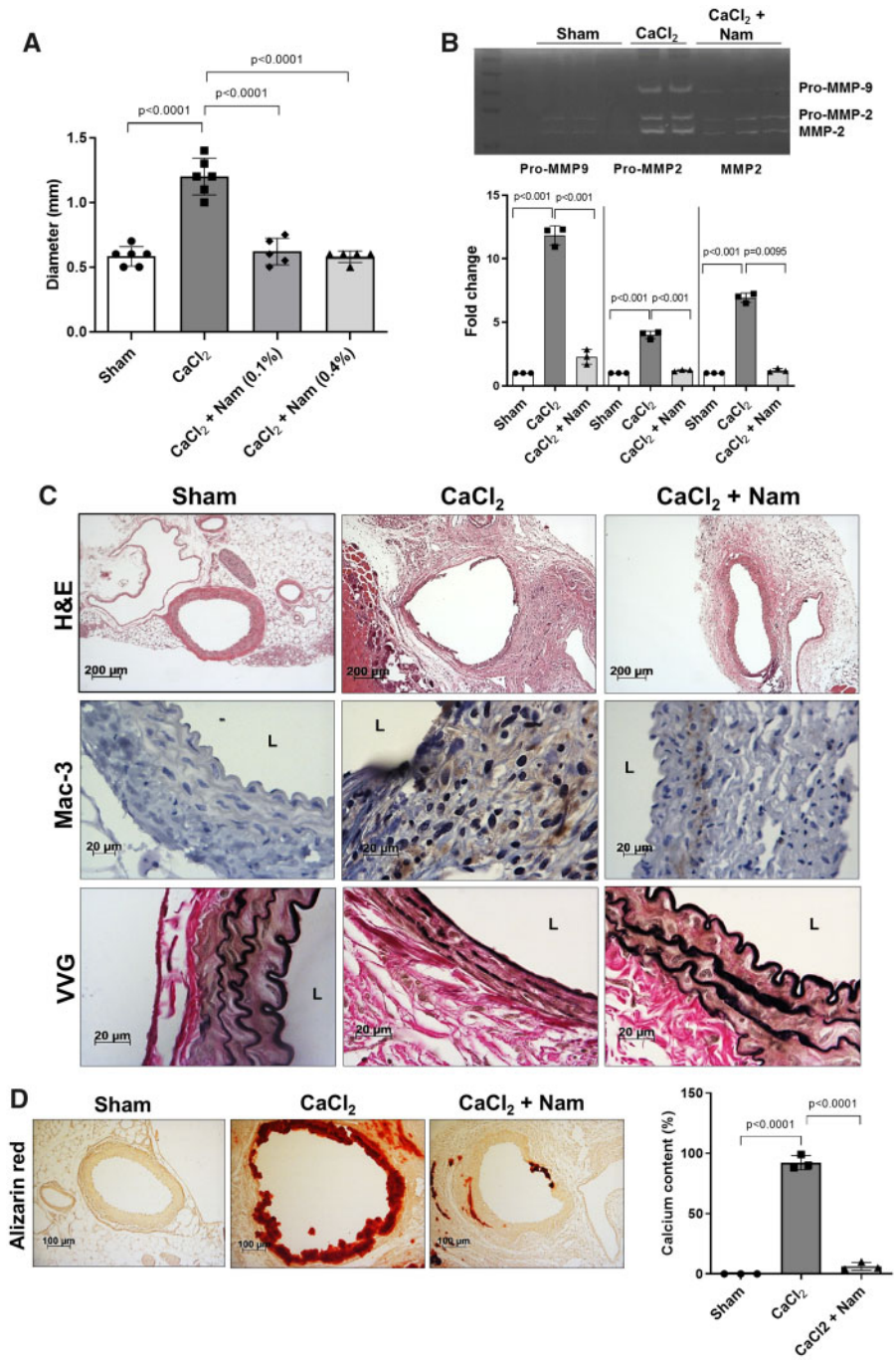


Figure 5 Nicotinamide inhibited $CaCl_2$-induced AAA formation. (*A*) AAA diameter ($n = 5–6$). (*B*) Representative zymogram (upper panel) and quantified data (bottom panel) for aortic MMP-2 and MMP-9 activities ($n = 3$). (*C*) Representative histology; H&E, Mac-3 (macrophages), and VVG (elastin fragmentation). (*D*) Alizarin red (left panel, aortic calcification) and quantified data (right, $n = 3$). Data were analysed using one-way ANOVA followed by Bonferroni *post hoc* analysis. $CaCl_2$, calcium chloride; H&E, haematoxylin & eosin; L, lumen; Mac, macrophage; Nam, nicotinamide; VVG, Verhoeff-van Gieson.

4.3 Sirt1 is a downstream regulator of NAD$^+$-mediated AAA protection

Sirtuins are NAD$^+$-dependent deacetylases that play various roles in cellular energy metabolism, DNA repair, inflammatory signalling, redox regulation, and regulation of transcription factors (i.e. p53, NFκB, FOXOs, PGC1α, and PARP1)[25,39] Interestingly, Sirt1 expression and activity are significantly decreased in human AAA tissues, and age-associated Sirt1 reduction in VSMCs increases susceptibility to AAA in mice.[40] Moreover, macrophage-specific Sirt1 expression was reported to contribute to AAA protection.[33] Sirt1 reduction also promoted vascular cell senescence, p21 up-regulation, and inflammatory cell recruitment.[33] In total, these studies suggest that Sirt1 reduction might be mechanistically

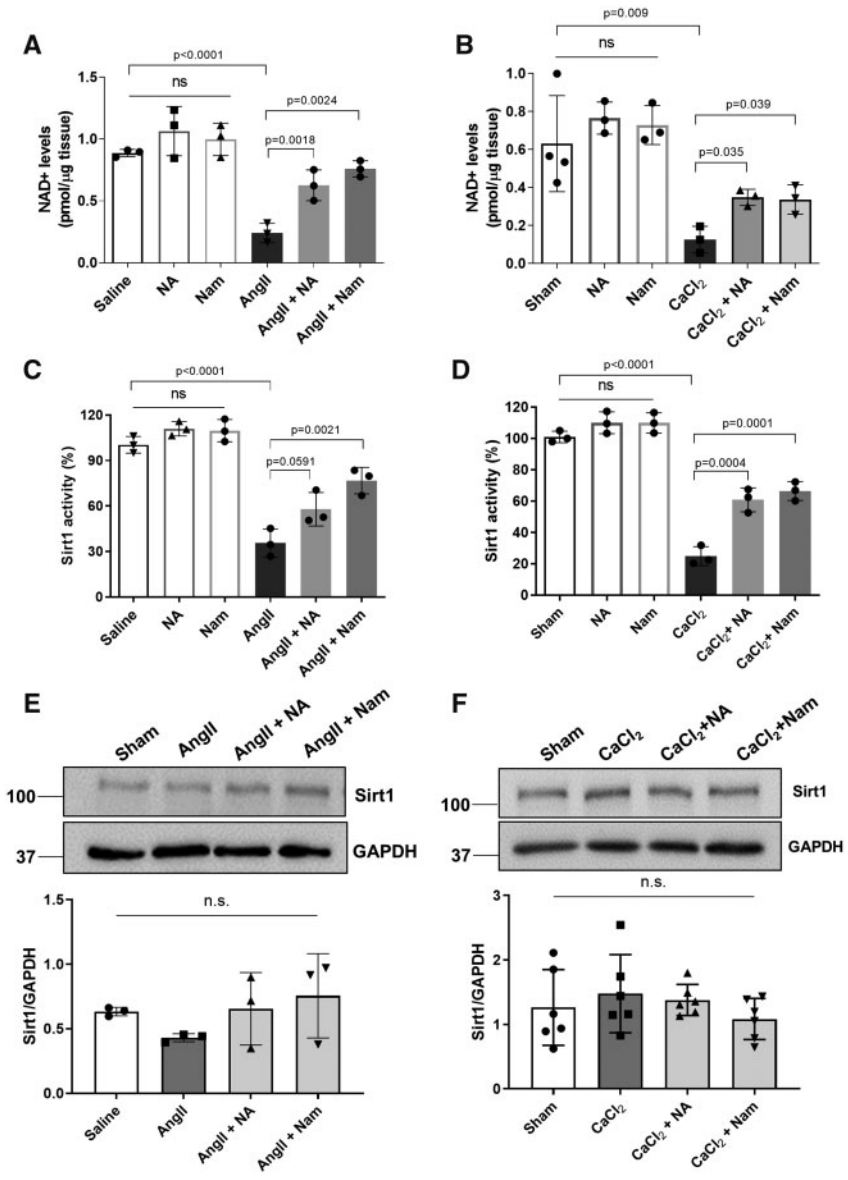


Figure 6 Aortic NAD^+ levels and Sirt1 activity were reduced in AAA and restored by either niacin or nicotinamide supplementation. Aortic NAD^+ levels in AngII-induced AAA model (*A*, $n = 3$) and $CaCl_2$-induced AAA model (*B*, $n = 3$). Sirt1 activities in AngII-induced AAA model (*C*, $n = 3$) and $CaCl_2$-induced AAA model (*D*, $n = 3$). Sirt1 protein expression levels in AngII-induced (*E*, $n = 3$) and $CaCl_2$-induced (*F*, $n = 6$) AAA models. Data were analysed using one-way ANOVA followed by Bonferroni *post hoc* analysis. AngII, angiotensin II; $CaCl_2$, calcium chloride; NA, nicotinic acid; NAD, nicotinamide adenine dinucleotide; Nam, nicotinamide; N.S., not significant; Sirt, sirtuin.

linked to AAA pathogenesis. Consistent with this notion, we observed reduced Sirt1 activity, but not protein expression, during experimental AAA formation. Notably, nicotinamide restored Sirt1 activity and protected against AAA formation, which was abrogated by a Sirt1 inhibitor, suggesting that Sirt1 is a downstream mediator of nicotinamide's effects. In this study, we employed a pharmacological Sirt1 inhibitor, EX-527, which potently and selectively inhibits Sirt1 activity (200–500-fold more potent against Sirt1 than Sirt2 or Sirt3) via a unique NAD^+-dependent deacetylation mechanism.[41] Mice in which Sirt1 has been genetically deleted have been developed, but inbred strains of these mice exhibited developmental defects and prenatal death,[42] while outbred mice led to viable but smaller mice with metabolic abnormalities.[43]

Our data suggest that nicotinamide prevents AAA formation by preserving Sirt1 activity, which can be blocker by the Sirt1 inhibitor EX-527. However, EX-527, when administered without nicotinamide, did not significantly augment AAA formation induced by either 1000 or 500 ng/kg/min of AngII (*Figure 7A*, Supplementary material online, *Figure S9*). The reason for these findings is unclear. However, it is noteworthy that Sirt1 activity was markedly suppressed (i.e. by approximately two-thirds) in the aortas of AngII-infused and $CaCl_2$-treated mice (*Figure 6C* and *D*).

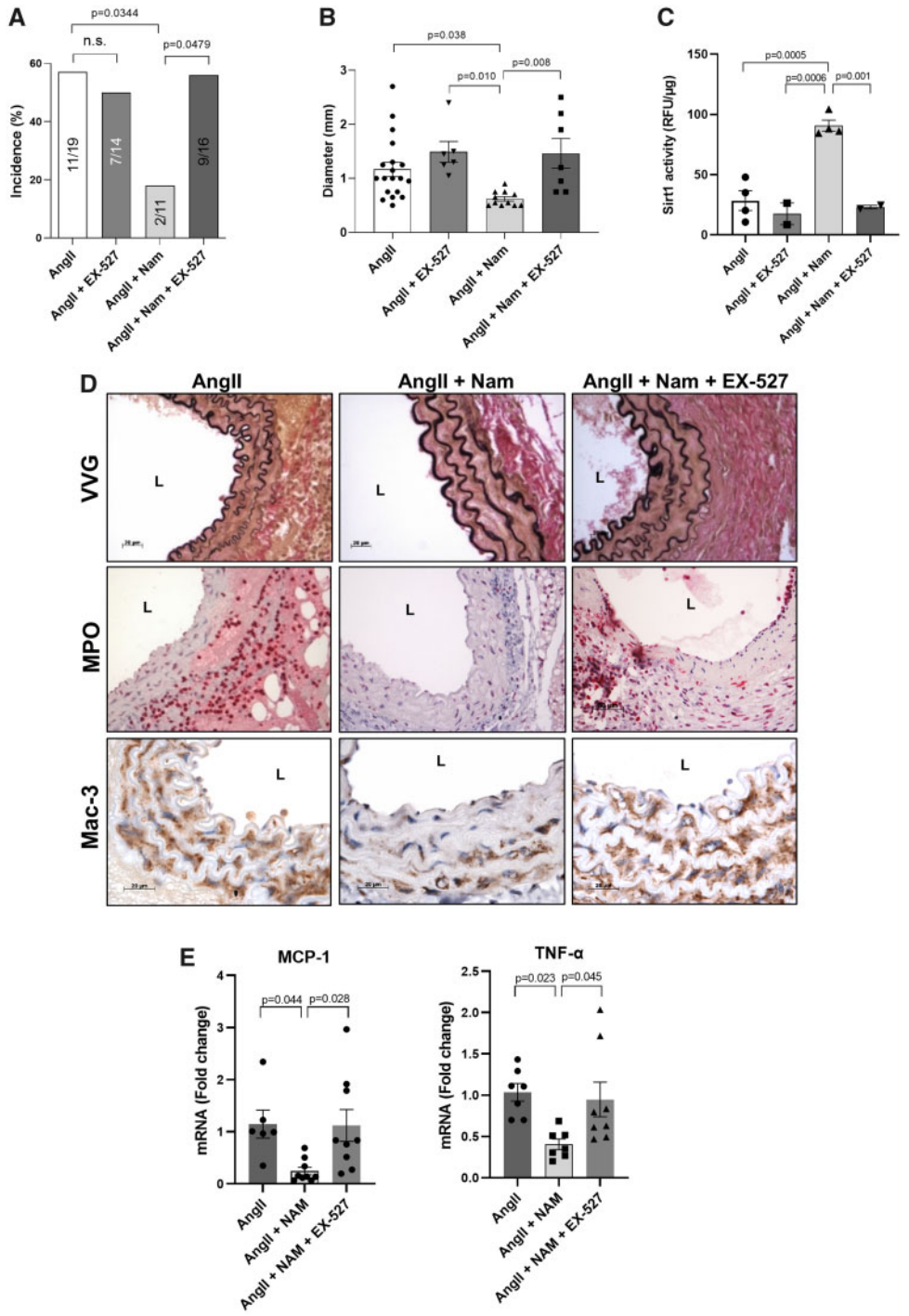


Figure 7 Protective effects of nicotinamide against AAA formation were abrogated by Sirt1 inhibition. AngII was infused via osmotic mini-pump in LDLR KO mice treated with or without nicotinamide ± EX-527, a selective Sirt1 inhibitor. (*A*) AAA incidence ($n = 11–19$). (*B*) Aortic diameter ($n = 6–19$). (*C*) Aortic Sirt1 activity ($n = 2–4$). (*D*) Representative histology; VVG (elastin fragmentation), MPO, and Mac-3 (macrophages). (*E*) Pro-inflammatory gene (MCP-1, left panel and TNFα, right, $n = 6–9$) expression. Data were analysed using one-way ANOVA followed by Bonferroni post-hoc analysis. AngII, angiotensin II; L, lumen; Mac, macrophage; MCP-1, monocyte chemoattractant protein 1; MPO, myeloperoxidase; Nam, nicotinamide; TNFα, tumour necrosis factor α; VVG, Verhoeff-van Gieson.

Under these conditions, it is reasonable to presume that an enzymatic inhibitor of Sirt1 might have little biological impact.

4.4 NAD+ precursors as novel AAA therapeutic agents?

Niacin, when added to statin therapy, failed to reduce atherosclerotic events in the AIM-HIGH and HPS2-THRIVE trials.[44,45] These data cannot be extrapolated to AAA, which is a distinct disease process from atherosclerosis. Considering that NAD+ repletion, rather than GPR109A activation, appears to account for niacin's beneficial effects against experimental AAA formation, our data suggest that nicotinamide or related biomolecules that elevate NAD+ levels yet lack the flushing side effects of niacin (which are specifically linked to GPR109A activation) merit further investigation. In this regard, several recent studies indicate that nicotinamide riboside is highly bioavailable, safe and effective at elevating NAD+ levels in adults.[46,47]

4.5 Study limitations

Although we used two different mouse models of AAA to investigate the role of niacin and nicotinamide in the pathogenesis of AAA, neither of these animal models perfectly mimics the pathology of AAA in humans. Moreover, while we demonstrated that niacin and nicotinamide can prevent AAA formation, we did not test their efficacy to limit the growth of existing AAA. Thus, while these findings should stimulate further research, they cannot be extrapolated to treatment of AAA in humans.

5. Conclusions

We provide novel evidence that niacin protects against AAA formation in two distinct animal models. Mechanistically, these effects are independent of GPR109A, shared by nicotinamide, and likely mediated through NAD+ repletion leading to Sirt1 activation. These results suggest that boosting NAD+ levels and Sirt1 activity by supplementation with nicotinamide or related biomolecules may represent an effective and well-tolerated approach to treating AAA.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Author's contributions

Conception and design of the study: A.L.B., N.L.W., and H.W.K. Performed experiments and collection of data: T.H., M.O., M.M., D.K., S.P., N.G., L.R., T.W.B., J.P., S.A., A.T., N.R., A.M., A.E., S.B., and H.W.K. Data analysis: T.H., A.L.B., M.O., S.P., N.G., T.W.B., A.T., N.R., A.M., A.E., S.B., N.L.W., and H.W.K. Interpretation of data and discussion: T.H., A.L.B., B.K.S., Y.H., D.J.F., G.A., N.S., S.O., N.L.W., and H.W.K. Manuscript preparation: A.L.B., N.L.W., and H.W.K.

Conflict of interest: none declared.

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Translational perspective

Abdominal aortic aneurysms (AAAs) are associated with pronounced adventitial and medial inflammation leading to matrix degradation and progressive aortic expansion. We report that niacin blunts aortic inflammation and matrix degradation, thereby suppressing AAA formation. These effects are independent of the niacin receptor GPR109A and mimicked by nicotinamide, which does not induce flushing. These results suggest that nicotinamide and related biomolecules that replete cellular NAD^+ may be an effective medical therapy for AAA.