RESEARCH ARTICLE

A cell permeant phosphopeptide mimetic of Niban inhibits p38 MAPK and restores endothelial function after injury

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Abstract

Vascular injury leads to membrane disruption, ATP release, and endothelial dysfunction. Increases in the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and decreases in the phosphorylation of Niban, a protein implicated in ER stress and apoptosis, are associated with vascular injury. A cell permeant phosphopeptide mimetic of Niban (NiPp) was generated. The effects of NiPp in restoring endothelial function were determined ex vivo using intact rat aortic tissue (RA) after pharmacological activation of p38 MAPK and also in multiple clinically relevant injury models. Anisomycin (Aniso) increased p38 MAPK phosphorylation and reduced endothelial-dependent relaxation in RA. Treatment with NiPp prevented Ansio-induced reduction in endothelial function and increases in p38 MAPK phosphorylation. NiPp treatment also restored endothelial function after stretch injury (subfailure stretch), treatment with acidic Normal Saline (NS), and P2X7R activation with 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate (BzATP). Aged, diseased, human saphenous vein (HSV) remnants obtained from patients undergoing coronary bypass surgical procedures have impaired endothelial function. Treatment of these HSV segments with NiPp improved endothelial-dependent relaxation. Kinome screening experiments indicated that NiPp inhibits p38 MAPK. These data demonstrate that p38 MAPK and Niban signaling have a role in endothelial function, particularly in response to injury. Niban may represent an endogenous regulator of p38 MAPK activation. The NiPp peptide may serve as an experimental tool to further elucidate p38 MAPK regulation and as a potential therapeutic for endothelial dysfunction.

KEYWORDS

endothelial dysfunction, kinase inhibitor, Niban, p38 MAPK, vascular

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Abbreviations: Aniso, anisomycin; BzATP, 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate; CCH, carbachol; HSV, Human saphenous vein; NiPp, Niban phosphopeptide mimetic; p38 MAPK, p38 mitogen-activated protein kinase; PE, phenylephrine; RA, rat aorta.

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1 | INTRODUCTION

The inner lining of the vascular wall consists of a monolayer of endothelial cells. Mechanical forces (blood flow disturbances, mechanical stretch),[1,2] chemical stressors (glycemic, oxidative, osmotic, acidosis),[3-9] inflammation,[10] and aging[11] are associated with dysfunction of this fragile endothelial monolayer. A common physiologic sequela of endothelial injury is impaired endothelial-dependent relaxation, often referred to as "endothelial dysfunction."

While multiple mechanisms have been implicated in promulgating endothelial injury, one of the common underlying themes is disruption of the endothelial membrane. Measurement of endothelial membrane injury has been performed by measuring extracellular release of biomolecules that have high intracellular concentrations. Adenosine triphosphate (ATP), in which there is a large gradient between intracellular (1-10 mM) and extracellular concentrations (1-10 μM), was one of the first markers of membrane injury.[12-14] More recently, lactate dehydrogenase (LDH) release has been used as a marker of membrane injury.[15,16] Loss of endothelial membrane integrity also leads to changes in transcellular resistance which can be measured with impedance (transepithelial/endothelial electrical resistance, TEER).[17] Exposure of veins to acidic saline solutions such as Normal Saline (NS) that is widely used as a resuscitation fluid, or mechanical stretch during surgical harvest leads to endothelial injury and release of ATP, LDH, and decreased TEER.[4]

Prolonged exposure to high concentrations of ATP activates the purinergic receptor, P2X7R. P2X7R activation is one of the most potent activators of the inflammasome.[18-20] P2X7R modulates responses to injury via activation of the p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway.[21-23] p38 MAPK is also activated by environmental stress and inflammatory cytokines.[24] p38 MAPK modulates a myriad of physiological processes through transcriptional regulation and/or activation of downstream kinases.[25] Thus, increased extracellular ATP after injury is not only a marker of injury, but may also play a role in propagating the vascular "response to injury."

A clinically relevant model of human vascular injury is the process of surgical harvest and preparation of human saphenous vein (HSV) prior to implantation as an autologous transplanted vascular graft.[3] HSV is injured by mechanical stretch during harvest and pressure distention, storage in acidic NS solution, and orientation marking with surgical skin markers.[1,4,9,22,26-28] Previously, to understand the response to surgical injury, segments of HSV removed atraumatically were compared to cognate segments after harvest and preparation injury. Injured HSV segments demonstrated impaired endothelial-dependent relaxation which was associated with a decrease in Niban phosphorylation.[22] The *Niban* gene, also known as FAM129A, was first identified as a gene upregulated in cancer.[29] Niban is involved in the regulation of cancer progression, cell proliferation, apoptosis, and endoplasmic reticulum (ER) stress responses.[30-33] Ji *et al* reported that Akt-dependent phosphorylation of Niban is involved in ultra-violet (UV)-induced cell apoptosis.[34] In Niban knockout mice, the ER stress response pathway was affected as phosphorylation of eukaryotic translational initiation factor (eIF) 2α, p70 ribosomal S6 subunit kinase (S6K) 1, and eukaryotic initiation factor 4E-bindingprotein (4E-BP) were altered, implicating a role of Niban in modulating translation in cell death signaling.[35] In a rat aorta (RA) model of subfailure stretch injury, decreased Niban phosphorylation was associated with an increase in p38 MAPK phosphorylation, supporting the interplay between p38 MAPK and Niban after acute vascular injury.[22] Taken together, these data suggest that Niban plays a protective role in response to cellular injury.

In this investigation, we sought to determine the relationship between p38 MAPK and Niban phosphorylation and determine the mechanistic interplay of these molecules that contributes to endothelial dysfunction. A cell permeant phosphopeptide mimetic of Niban (NiPp) were designed, synthesized, and characterized to function as an experimental tool as well as a potential therapeutic approach to treating endothelial dysfunction.

2 | MATERIAL AND METHODS

2.1 | Materials

All chemicals and reagents were purchased from Sigma unless otherwise described. The peptide (NiPp) used in this study was synthesized by f-moc chemistry and purified using high-performance chromatography by EZBiolab (NJ).

2.2 | Tissue procurement

Aorta (RA) was procured from 250-300 g, Sprague Dawley rats. Animal procedures followed study protocols approved by the Vanderbilt Institutional Animal Care and Use Committee and adhered to National Institute of Health guidelines for care and use of laboratory animals. Immediately after euthanasia by CO_2, the thoracic and abdominal RA was isolated via an incision along the mid-abdomen, placed in heparinized PlasmaLyte (PL; 10 unit heparin/mL PlasmaLyte, Baxter, Deerfield IL) and transported to the laboratory for immediate testing.

HSV was obtained under approval from the Institutional Review Board of Vanderbilt University Medical Center from consented patients undergoing coronary artery bypass grafting procedures. HSV segments were collected immediately following surgical harvest and transported to the laboratory in PL for immediate experimentation.

2.3 | Measurement of endothelial-dependent relaxation

Rings of HSV or RA (1-2 mm) were suspended in a muscle bath containing a bicarbonate buffer (120 mM sodium chloride, 4.7 mM potassium chloride, 1.0 mM magnesium sulfate, 1.0 mM monosodium phosphate, 10 mM glucose, 1.5 mM calcium chloride, and 25 mM sodium bicarbonate, pH 7.4) equilibrated with 95% O2/5% CO2 at 37°C for 1 hour at a resting tension of 1 g, manually stretched to three times the resting tension, and maintained at resting tension for an additional 1 hour. This produced the maximal force tension relationship as previously described.[4] After equilibration, the rings were primed with 110 mM of potassium chloride (with equimolar replacement of sodium chloride in bicarbonate buffer) to determine functional viability. Viable rings were then tested for contractile response to a dose of phenylephrine (PE) to yield submaximal contraction (approximately 60%-70% of maximum KCl; 5×10^{-6} M for HSV and $1\text{-}5 \times 10^{-7}$ M for RA) and relaxed with carbachol (CCH, 5×10^{-7} M), an acetylcholine analogue, to determine endothelial-dependent relaxation responses. Force measurements were obtained using the Radnoti force transducer (model 159901A, Radnoti LLC, Monrovia, CA) interfaced with a PowerLab data acquisition system and LabChart software (AD Instruments Inc, Colorado Springs, CO). Contractile responses were defined by stress, calculated using force generated by tissues as follows: stress ($\times 10^5$ N/m^2) = force (g) $\times$ 0.0987/area, where area = wet weight (mg)/at maximal length (mm)]/1.055. Relaxation was calculated as percent change in stress compared to the maximal PE-induced contraction (set as 100%). Each data point was averaged from at least two rings from the same specimen. To determine concurrent signaling events, tissues were frozen in liquid nitrogen at relevant timepoints.

2.4 | p38 MAPK activation with Anisomycin

To activate p38 MAPK, thoracic RA were cut into rings and incubated in PL either alone or with anisomycin (Aniso) (200 μM), or pretreated with NiPp (500 μM) for 30 minutes followed by Aniso (200 μM) for 1 hour. After treatments, RA were either suspended in the muscle bath to determine endothelial-dependent relaxation or snap-frozen for Western blot analysis.

2.5 | Vascular injury and treatment of RA

Abdominal RA was subjected to subfailure stretch to the haptic endpoint (~200% the resting length) for 10 s and repeated twice as previously described [36] and a segment was reserved as non-stretched control. The tissues were then cut into 1-2 mm rings and incubated for 1 hour at room temperature in PL with or without NiPp (500 μM).

To induce acidotic injury, thoracic RA were cut into 1-2 mm rings and pretreated in the absence or presence of NiPp (500 μM) in PL for 30 minutes. Tissue rings were then transferred to NS and continued incubation in the absence or presence of NiPp (500 μM) for 2 hours. Control rings were incubated in PL for 2.5 hours.

To induce P2X7R associated endothelial dysfunction, thoracic RA rings were incubated with the P2X7R agonist 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate (BzATP; 1 mM) in the absence or presence of NiPp (500 μM) in PL for 1 hour at room temperature.

After treatments, RA were either suspended in the muscle bath to determine endothelial-dependent relaxation or snap-frozen for Western blot analysis.

2.6 | Culture and treatment of human coronary artery endothelial cell

Human coronary artery endothelial cell (HCAEC) were obtained from Cell Applications (San Diego, CA) and grown in Human Meso Endo Growth Medium (Cells Applications) maintained in a 37ºC and 5% CO$_2$ incubator and were passaged at 80% confluence. Cells between passage 5-6 were used.

HCAEC were plated in 60 mm dishes at 70%-80% confluency. After serum-deprivation in culture medium diluted with 90% of basal medium for 3 hour, cells were either untreated or treated with NiPp (100 μM) for 1 hour prior to BzATP (1 mM) treatment for an additional 1 hour. Cells were washed and lysed with a modified RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 1 mM EDTA, 1 mM EDTA) supplemented with protease and phosphatase inhibitors.

2.7 | Western blot analysis

Frozen tissues were pulverized, and proteins were extracted in the modified RIPA buffer. Proteins from tissue or cell lysates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane followed by immunoblotting with the antibodies against phospho-p38 MAPK-Thr180/Tyr182, p38 MAPK, phospho-JNK-Thr183/Tyr185, JNK, phospho-ERK-Thr202/Tyr204, ERK (Cell Signaling Technology, CA), and GAPDH (Millipore, MA) or tubulin (Sigma, MA) for protein loading control. The blots were then incubated with IRDye labeled secondary antibodies (LI-COR Biosciences, NE). The protein-antibody complexes were visualized and quantified using the Odyssey Infrared Imaging System. Phosphorylation was calculated as a ratio of the phosphorylated protein to

total protein and was then normalized to the untreated tissues (Ctrl) with the control value set as 1.0.

2.8 | Kinase profiling

NiPp was dissolved in DMSO, screened at a single concentration of 100 µM *using* in vitro kinase assays (SelectScreen Kinase Profiling Service; Thermo Fisher Scientific, Madison WI) including the Z'LYTE and Adapta kinase activity assays and the LanthaScreen Eu Kinase Binding Assay). Candidate interactions were predicted using STRING Version 11 (www.string-db-org) with the human database at confidence = 0.7.

2.9 | Statistical analysis

Data were reported as mean responses ± standard deviation. Outliers, normality, and statistical significance (*P* value) were determined using GraphPad Prism version 5.0. Differences between two groups were determined by paired *t* test for experiments with dependent (matched) pairs. One-way or two-way ANOVA with post hoc tests were used to determine differences among multiple, dependent (matched) samples from the same animal. A *P* value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Design of phosphomimetic of Niban, NiPp

Phosphorylation of Niban at serine 602 is downregulated during UV-induced cell death [34] and injured vascular tissues.[22] A peptide (Niban phosphopeptide, "NiPp") was designed to contain the enhanced protein transduction domain TAT,[37,38] conjugated to a phosphopeptide analog of the region surrounding serine 602 of Niban (YARAAARQARA**SPAARRAS*AILPG;** bold = Niban sequence; S*=phosphoserine). Multiple batches were synthesized and displayed similar bioactivity (Supporting Figure S1).

3.2 | Anisomycin treatment activates p38 MAPK and impairs endothelial function of intact rat aortic tissues

Intact strips of RA were treated with Aniso (Figure 1A,B), an antibiotic produced by *Streptomyces griseolus* known to induce p38 MAPK activation in endothelial cells.[39] Anisomycin treatment led to increases in the phosphorylation of p38 MAPK (Figure 1C,D, and Supporting Figure S2)

and impaired endothelial-dependent relaxation (87.3 ± 5.4 vs 50.6 ± 12.2%; Figure 1B).

3.3 | NiPp restored endothelial function in anisomycin-treated RA and reduced p38 MAPK phosphorylation

Using the model of impaired endothelial-dependent relaxation in Aniso-treated vessels, RA was treated with buffer alone, Aniso or NiPp for 30 minutes followed by Aniso. Pretreatment with NiPp improved endothelial-dependent relaxation (50.6 ± 12.2 vs 64.9 ± 10.5%) and decreased Aniso-induced increases in p38 MAPK phosphorylation (Figure 1 and Supporting Figure S2). Anisomycin also activates c-Jun N-terminal kinases (JNK) which in turn may cross-talk with extracellular signal-regulated kinases (ERK). NiPp pretreatment did not reduce JNK or ERK phosphorylation in Aniso-treated RA (Figure 2 and Supporting Figure S3).

3.4 | NiPp restored endothelial function and reduced P38 MAPK phosphorylation after subfailure stretch injury in RA

To determine if NiPp restores endothelial function after other types of injury, RA was subjected to stretch injury [36] and treated with NiPp. NiPp partially restored endothelial function after stretch injury (43.5 ± 7.9 vs 28.9 ± 6.9% at 5×10^{-7}M CCH; Figure 3A) and reduced p38 MAPK phosphorylation (Figure 3B,C and Supporting Figure S4).

3.5 | NiPp restored endothelial function after acidosis injury and reduced P38 MAPK phosphorylation in RA

An additional, clinically relevant type of injury is exposure to acidic NS solution commonly used clinically for intravenous resuscitation and for storage of vascular tissues prior to use as autologous vascular reconstruction conduits.[40-42] Pretreatment with NiPp improved endothelial function in RA incubated in NS (74.1 ± 7.6 vs 51.4 ± 18.4% Figure 4A) and reduced p38 MAPK phosphorylation (Figure 4B and Supporting Figure S5).

3.6 | NiPp restored endothelial function after P2X7R activation in RA and reduced p38 phosphorylation in cultured human endothelial cells

Both stretch and NS-induced injury lead to release of ATP and activation of P2X7R.[4,22] RA were treated with the ATP
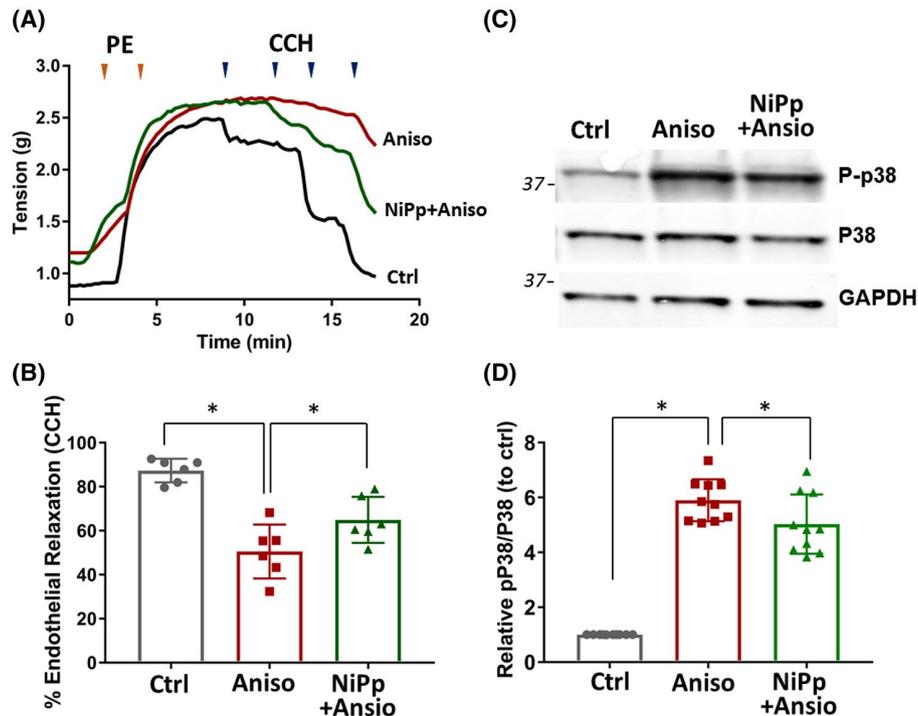
FIGURE 1 Anisomycin-induced phosphorylation of p38 MAPK and decreased carbachol (CCH)-induced relaxation in rat aorta (RA) is partly prevented by treatment with Niban peptide (NiPp). RA rings were suspended in a muscle bath and incubated with either buffer alone (Ctrl), Aniso (200 μM) for 1 h, or NiPp (500 μM) for 30 min followed by Aniso (200 μM) for 1 h. A, RA were suspended in the muscle bath, contracted with PE, and then, relaxed with escalating doses of CCH (10^{-8} to 10^{-5} M). The force generated was determined and was adjusted to the weight and length of the tissue. Representative tracings from 1 out of 6 different rats are shown. Orange arrow heads indicate addition of PE (orange) and CCH (blue). B, Percent relaxation induced by 5×10^{-7} M CCH was calculated. $*P < .05$ in one-way ANOVA with Tukey posttest. RA rings were snap-frozen either untreated or immediately after treatment. Proteins were extracted and phosphorylation of p38 MAPK was determined by Western blot analysis. C, Western blots shown are representative of 1 out of 10 rats. D, Quantification of relative phosphorylation to total p38 MAPK level. n = 10, $*P < .05$ in one-way ANOVA with Tukey posttest. Data are expressed as mean ± SD

analogue, BzATP, a potent and specific P2X7R agonist in the presence of NiPp. NiPp co-treatment prevented endothelial dysfunction induced by BzATP (74.4 ± 11.1 vs 44.2 ± 19.6%; Figure 5A) but did not reduce p38 phosphorylation (data not shown). Since the potent BzATP-induced activation of the P2X7R-p38 MAPK signaling likely occurred in both endothelial and smooth muscle layers in intact tissue, primary human coronary aorta endothelial cells were treated with BzATP to determine if NiPp reduces p38 MAPK specifically in endothelial cells. BzATP-induced p38 MAPK phosphorylation was reduced by NiPp treatment (Figure 5B and Supporting Figure S6).

3.7 | NiPp improved endothelial function in human saphenous vein harvested for coronary artery bypass surgery

To determine the effect of NiPp on human tissues with endogenous impaired endothelial function, segments of HSV were harvested at the time of coronary artery bypass surgery. The tissues were either untreated or incubated in the presence of NiPp for 2 hours and endothelial responses were determined.

Treatment with NiPp improved endothelial-dependent relaxation (12.1 ± 7.3 vs 6.9 ± 6.9% Figure 6).

3.8 | NiPp is a kinase inhibitor

As injury leads to increased p38 MAPK phosphorylation and decreased Niban phosphorylation,[22] one of the possible mechanisms by which NiPp restores function after endothelial injury is kinase inhibition. A kinase profiling was performed using three different profiling platforms that measure activity and kinase/substrate binding. Kinases of which activity or substrate binding were inhibited by NiPp (100 μM) at > 40% are listed and shown in a kinase dendrogram (Figure 7). Full data set containing all 490 kinases is presented in Supporting Data S1.

The top two candidate kinase targets of NiPp were mitogen- and stress-activated kinase 1 (MSK1; also known as RPS6KA5) and p38 MAPKα (MAPK14), which were inhibited by 66% and 61%, respectively. MSK1 is an AGC kinase of the RSK family that is phosphorylated by ERK and p38 MAPK in response to cellular stress. Other kinases inhibited
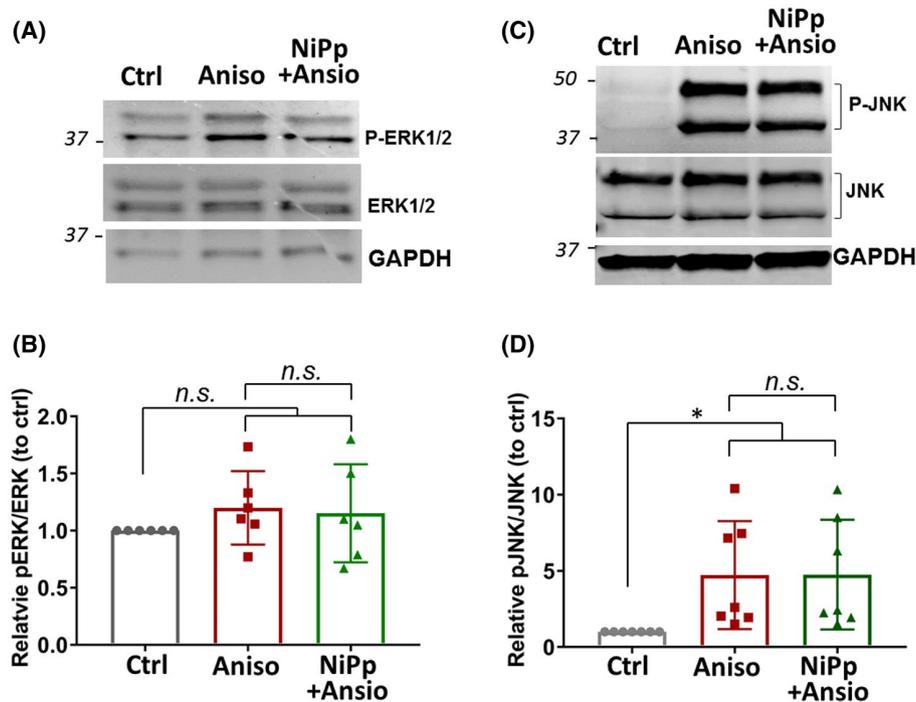
FIGURE 2 NiPp does not affect ERK or JNK phosphorylation in anisomycin-treated rat aorta (RA). RA rings (n = 6-7 rats) were suspended in a muscle bath and incubated with either buffer alone (Ctrl), Aniso (200 μM) for 1 h, or NiPp (500 μM) for 30 min followed by Aniso (200 μM) for 1 h. Tissues were then snap-frozen and protein extracted to determine ERK and JNK by Western blot analysis. A and C, Western blots shown are representative of 1 out of 6-7 rats. B and D, Quantification of relative phosphorylation to total ERK or JNK levels, *P<.05 in one-way ANOVA with Tukey posttest comparing matched treatment groups of the same animal. Data are expressed as mean ± SD
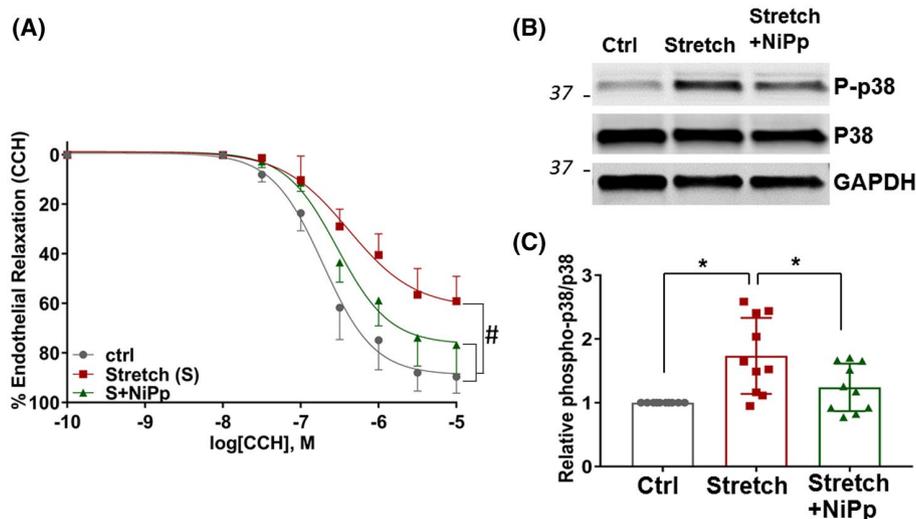

FIGURE 3 NiPp partially restores endothelial function and reduces p38 MAPK phosphorylation in rodent aortic tissue after subfailure stretch injury. Freshly isolated rat aorta (RA) was subjected to subfailure stretch and incubated in PL in the absence (S) or presence of NiPp (500 μM) for 1 h at room temperature. A, RA (n = 5 rats) were either suspended in the muscle bath, contracted with PE, and then, treated with escalating doses of carbachol (CCH; 10^{-8} to 10^{-5} M). The percent relaxation was determined as a change to the maximal PE-induced contraction. #P<.05, two-way ANOVA comparing matched treatment groups of the same animal. B, RA (n = 10 rats) were snap-frozen after stretch injury and treatment with NiPp (500 μM) for 1 h, protein extracted and immunoblotted to examine p38 MAPK phosphorylation. C, Western blots shown are representative of 1 out of 10 rats. D, Quantification of relative phosphorylation to total p38 MAPK level. *P<.05, in one-way ANOVA with Tukey posttest comparing matched treatment groups of the same animal. Data are expressed as mean ± SD

(A)

(B)

(C)
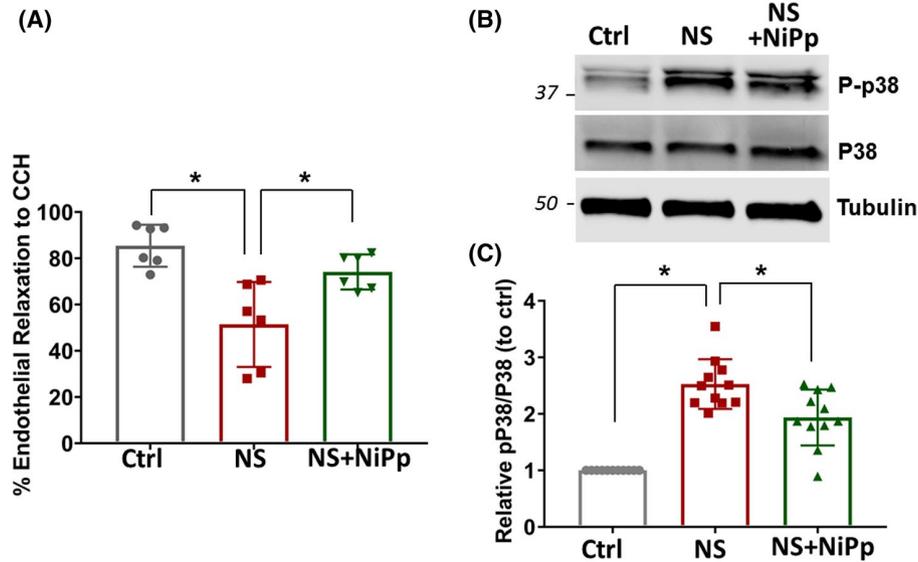
FIGURE 4 NiPp partially restores endothelial function in rodent aortic tissues after acidosis injury. Freshly isolated rat aorta (RA) was cut into rings, and then, pretreated in the absence or presence of NiPp (500 μM) in PL for 30 min. Tissue rings were then transferred to NS and continued incubation in the absence or presence of NiPp (500 μM) for 2 h at room temperature. Control rings (Ctrl) were incubated in PL for 2.5 h. A, After treatments, RA (n = 6 rats) were suspended in the muscle bath, contracted with PE, and then, treated with escalating doses of carbachol (CCH; 10^{-8} to 10^{-5} M). The percent relaxation was determined as a change to the maximal PE-induced contraction. Percent relaxation to 5×10^{-7} M CCH is shown. *$P < .05$ in one-way ANOVA with Tukey posttest. Data are expressed as mean ± SD B, RA (n = 11 rats) were snap-frozen after treatment, protein extracted and immunoblotted to examine p38 MAPK phosphorylation. C, Western blots shown are representative of 1 out of 11 rats. D, Quantification of relative phosphorylation to total p38 MAPK level. *$P < .05$, in one-way ANOVA with Tukey posttest comparing matched treatment groups of the same anima. Data are expressed as mean ± SD

(A)

(B)

(C)
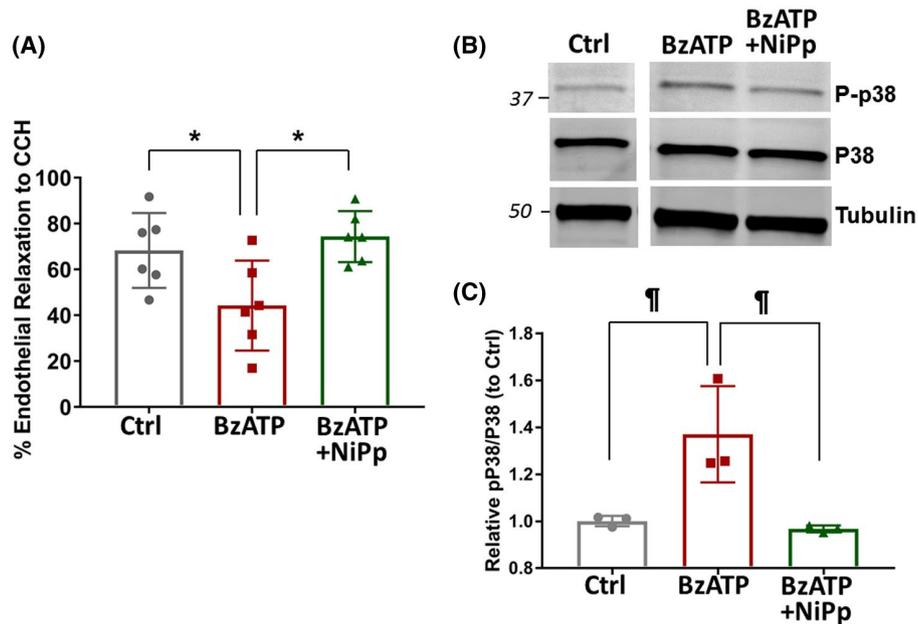
FIGURE 5 NiPp restores endothelial function in rodent aortic tissues and reduces p38 MAPK phosphorylation in primary endothelial cells after P2X7R activation. Freshly isolated rat aorta (RA) was cut into rings and either left untreated (Ctrl) or treated with BzATP (1 mM) in the absence of presence NiPp (500 μM) in HP for 2 h at room temperature. A, After treatments, RA were suspended in the muscle bath, contracted with PE, and then, treated with escalating doses of carbachol (CCH; 10^{-8} to 10^{-5} M). The percent relaxation was determined as a change to the maximal PE-induced contraction. Percent relaxation to 5×10^{-7} M CCH is shown. n = 6 rats. *$P < .05$ in one-way ANOVA with Tukey posttest comparing matched treatment groups of the same animal. B, Primary human coronary artery endothelial cells (n = 3) were either left untreated or treated with NiPp (100 μM) prior to BzATP (1 mM) treatment. Proteins were extracted and immunoblotted to examine p38 MAPK phosphorylation. B, Western blots shown are representative of 1 out of 3 biological replicates. C, Quantification of relative phosphorylation to total p38 MAPK level. ¶ $P < .05$ in unpaired one-way ANOVA with Tukey posttest. Data are expressed as mean ± SD
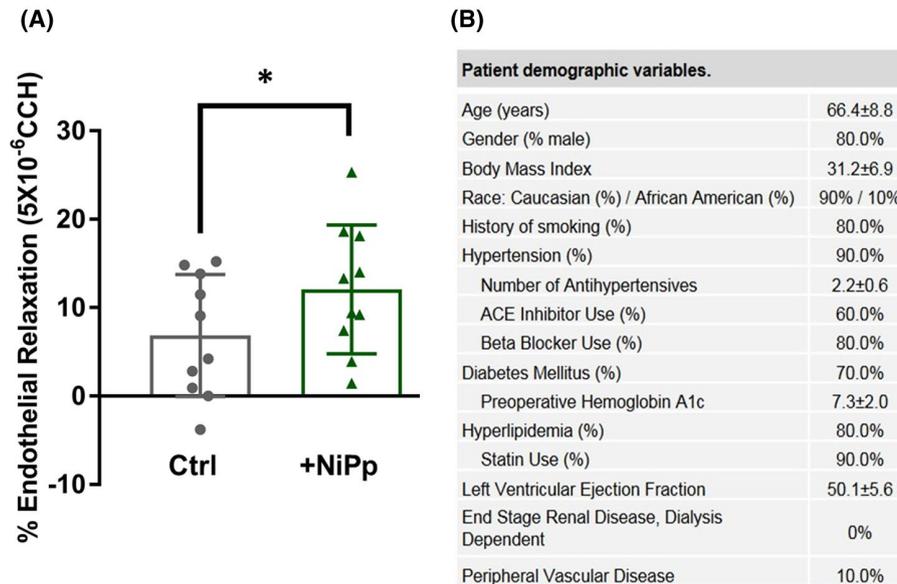
FIGURE 6 NiPp improves endothelial relaxation in human saphenous veins (HSV). A, HSV, collected from patients undergoing CABG immediately after surgical harvest, were either incubated in PL in the absence (Ctrl) or presence of NiPp (100 µM) for 2 h at room temperature. HSV were suspended in the muscle bath, contracted with PE and treated with carbachol (CCH; 10^{-8} to 10^{-5} M). The percent relaxation was determined as a change to the maximal PE-induced contraction. Percent relaxation to 5×10^{-6} M CCH is shown. n = 10; *$P<.05$ in paired t test. B, Patient demographic variables. Data are expressed as mean ± SD

4 | DISCUSSION

Previous findings that p38 MAPK activation after vascular injury is associated with endothelial dysfunction and decreased Niban phosphorylation suggest a potential link between the two proteins[1,22] and led to the hypothesis that restoration of Niban phosphorylation may improve endothelial function. In this study, we demonstrated that pharmacological activation of p38 MAPK with Aniso was modulated by NiPp, a phosphopeptide mimetic of Niban. NiPp pretreatment reduced p38 MAPK phosphorylation and prevented endothelial dysfunction, implicating that NiPp may ameliorate injury responses that involve p38 MAPK signaling. p38 MAPK is activated in response to multiple cellular stressors including infection, UV exposure, and ischemic injury of the brain, kidney, liver, and heart. p38 MAPK also responds to inflammatory cytokines and is a key mediator of inflammatory responses.[43] In the vascular wall, p38 MAPK is activated following balloon or bypass grafting-related injuries and promotes neointima formation.[44,45] Niban is characterized as an ER stress-related, antiapoptotic protein. A number of studies demonstrate that ER stress plays a role in endothelial

by the NiPp include CKD18 of the CMGC kinase family), FGFR1 of the TK family, and the PIK3R3 kinase (Figure 7). Kinase interaction network analyses of kinase candidates (Supporting Figure S7) and details on functional enrichment of this network is presented in Supporting Data S2.

dysfunction and leads to p38 MAPK activation.[46] Acidosis, P2X7R activation and mechanical stretch are also known to induce ER stress signaling in various cell types.[47-49] In this investigation, impaired endothelial responses caused by these injuries in RA lead to increases in p38 MAPK phosphorylation,[1,4] and all of which were improved with NiPp treatment. These results further support roles for p38 MAPK and Niban in acute vascular injury response.

Impaired endothelial function is associated with aging, atherosclerosis, diabetes, and renal failure.[50,51] Saphenous vein harvested for coronary reconstructions were obtained from an aged patient population (66.4 ± 8.8 years old) with systemic atherosclerosis and multiple comorbidities (Figure 4B). HSV obtained from this population typically displays impaired endothelial-dependent relaxation (10%-15% at 5×10^{-7}M CCH)[3,4,9] compared to normal healthy tissues from young animals such as pigs or rats (~50%-70%; Figures 1,3-6, Supporting Figure S8).[4,26] Thus, HSV represents a model of endogenous endothelial dysfunction in human tissue. Treatment of HSV with NiPp resulted in improvement of endothelial-dependent relaxation (Figure 6). Given that injury to HSV is associated with decreases in Niban phosphorylation,[22] this finding suggests that Niban may be involved in cellular signaling events that regulate the response to vascular injury and that NiPp may be used to improve endothelial function in diseased human tissues.

The mechanism by which NiPp restored endothelial function after injury is not known. Since NiPp treatment was associated with decreased phosphorylation of p38 MAPK in

(A)

Kinases	% Inhibition
MSK1 (RPS6KA5)	66
p38MAPKalpha	61
CDK18/cyclin Y	46
FGFR1	43
PIK3CA/PIK3R3	40

(B)
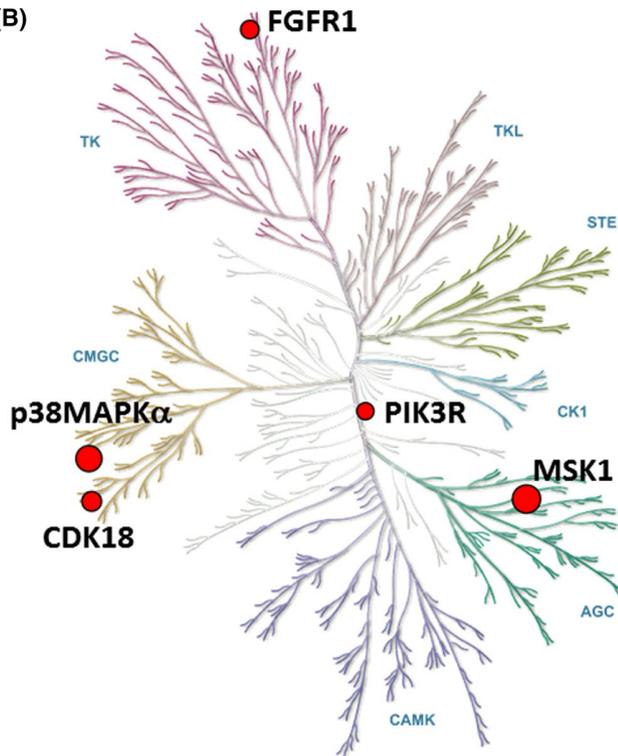
FIGURE 7 Kinase profiling of NiPp. NiPp was profiled against 490 kinases at 100 μM using the SelectScreen Kinase Profiling Service (www.thermofisher.com) as described in Materials and Methods. A, Candidates showing more than 40% inhibition. B, Kinase dendrogram (adapted with permission from Cell Signaling Technology) showing proportional circle to % inhibition using KinMap (www.kinhub.org)

RA, a potential mechanism of NiPp function is kinase inhibition. Therefore, a kinase profiling assay was performed to determine the effect of NiPp on the activity of a panel of kinases in vitro. The top two kinases inhibited by NiPp were MSK1 and p38 MAPKα. Inhibition to the p38 MAPKα isoform appeared to be specific as inhibition by NiPp to the other isoforms (β, δ, and γ) in the kinase profiling screen were only at 5, 3, and 3%, respectively (Supporting Data S1). Despite high sequence homology, the isoforms have difference in tissue expression, upstream kinase activators, and downstream substrates. The p38 MAPKα isoform, initially identified as a protein that underwent phosphorylation in response to endotoxin treatment and hyperosmolarity shock,[52] plays a role

in endothelial dysfunction in that inhibition of this isoform leads to improved endothelial function in animal models of cardiovascular diseases including cardiac hypertrophy, balloon injury, salt/fat-induced hypertension and in hypercholesterolemic patients.[53-56] The α-isoform is also a key regulator of pro-inflammatory cytokine production[25] and itself can be activated by IL1-β.[57] MSK1, a downstream kinase of the p38 MAPK activation (Supporting Figure S2),[58] has complex cell-dependent roles in inflammatory responses. In endothelial cells, MSK1 promotes CREB activation in response to TNFα.[59] Interestingly, *Niban* expression is also upregulated by IL1-β and has been implicated in steroid-responsive inflammatory responses in asthma.[60,61] Further analyses of the STRING database revealed that the candidate targets of NiPp are involved in 39 KEGG pathways that are implicated in cancer development, stress responses, and cytoskeletal regulation (Supporting Data S2). In addition, kinase screening data showed that NiPp does not inhibit ERK1/2 (4 and 6%, respectively) or JNK1/2/3 (8, 12, and 8%, respectively) in vitro (Supporting Data S1) and NiPp treatment had no effect on phosphorylation of ERK and JNK after Aniso treatment (Figure 2). These data highlight the potential of Niban as a stress response protein that participates in cellular injury responses.

While p38 MAPK activation plays a role in the response to injury, subsequent downregulation after injury is necessary to restore cellular homeostasis, uncontrolled p38 MAPK responses may contribute to aberrant downstream p38 MAPK-dependent signaling. Activation of p38 MAPK occurs via phosphorylation of the Thr-Gly-Tyr motif by upstream MKKKs and MKK3/MKK6.[25] Typically, protein phosphatases interact with kinases to downregulate activation. More recently, microRNAs were also reported to regulate p38 MAPK.[62] A number of protein phosphatases have been identified to carry out this function on p38 MAPK. The finding that NiPp reduces p38 MAPK phosphorylation and restores function after vascular injury raises the possibility that Niban may be an endogenous downregulator of p38 MAPK that restores cellular homeostasis after injury. This is consistent with some of the known functions of Niban as a stress responsive molecule.[34,35,63] Very few endogenous kinase inhibitors have been identified and characterized to date. Protein kinase inhibitor (PKI), which inhibits Protein Kinase A, is an anti-inflammatory and antiproliferative protein regulator in endothelial and vascular smooth muscle cells, respectively.[64,65] Another endogenous kinase inhibitor, secretoneurin, inhibits Calcium/Calmodulin-Dependent Protein Kinase II, and attenuates calcium-dependent arrhythmias as well as playing a critical role in neural vasculature.[66,67] Thus, while few endogenous kinase inhibitors have been identified, they do exist and phosphorylated Niban may, at least in part, modulate cellular responses via p38 MAPK inhibition. Whether Niban functions upstream or downstream of p38 MAPK or a p38

MAPK-Niban feedback regulatory circuit exists has yet to be determined.

p38 MAPK has been a prime target for the development of small molecule therapeutics; however, given its central role in many organs, toxicity has been a major limitation possibly due to the crosstalk between different intracellular pathways that p38 MAPK regulates. The finding that NiPp possesses an isoform-specific inhibitory property to p38 MAPK may offer an opportunity to develop a therapeutic that mimics endogenous signaling, possibly in a tissue specific manner. Moreover, NiPp modulation of the p38 MAPK signaling pathway may attenuate injury responses that occur during traumatic injuries such as surgery, sepsis, or inflammatory diseases. In addition, the unique approach of utilizing a cell permeant peptide analogue of phosphorylated Niban to elucidate the function of this molecule and its role in p38 MAPK signaling cascade is relevant to intact vascular and other tissues where genetic engineering approaches are less optimal due to low cellular turnover.

5 | LIMITATIONS

Contractile profiles of human saphenous veins were heterogenous due to varied patient demographics and may impart different responses to peptide treatments; however, paired untreated segments served as the control for comparison to the NiPp treated response. The kinase profiling was an in vitro study, validation of kinase targets will need to be performed in in vivo systems in future studies. These studies demonstrated that endothelial dysfunction to multiple types of injury and in different tissues could be restored with NiPp; however, future studies with scrambled cargo sequence peptides and peptides without CPP will confirm specificity of these responses.

6 | CONCLUSION

Vascular injury activates the p38 MAPK signaling cascade leading to endothelial dysfunction with concomitant decreases in Niban phosphorylation. A novel phosphopeptide mimetic of Niban was developed and function, at least in part by inhibiting p38 MPAK phosphorylation and restoring endothelial function in response to injury. These findings suggest that an association between p38 MAPK and Niban may play a crucial role in modulation of the injury response that is central to many vascular pathological conditions. Moreover, Niban may be an endogenous inhibitor of p38 MAPK. Synthetic small molecule inhibitors of p38 MAPK inhibitors have been limited in their therapeutic use due to issues with toxicity, solubility, and tachyphylaxis. A molecular mimic of an endogenous inhibitor of p38 MAPK may be less toxic and

have greater therapeutic potential as a biological approach to ameliorate the stress response to injury.

CONFLICT OF INTEREST
The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

AUTHOR CONTRIBUTIONS
P. Komalavilas, C. Brophy, and J. Cheung-Flynn designed research; TW Yim, D. Perling, M. Polcz, P. Komalavilas, and J. Cheung-Flynn performed research; TW Yim, D. Perling, P. Komalavilas, and J. Cheung-Flynn analyzed data; C. Brophy, and J. Cheung-Flynn wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.