

Bromelain enhances nitric oxide bioavailability: Bradykinin's link to TRPV1/Ca²⁺ /AMPK/autophagy signaling

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ABSTRACT

Bromelain, a protease enzyme extracted from the pineapple stem, is suggested to protect against atherosclerosis, non-alcohol fatty liver diseases, and coagulation dysfunction. However, the mechanism underlying the vascular protection of bromelain in the cardiovascular system is not fully understood. In this study, we explored the role of the kininogen-bradykinin system in bromelain-mediated nitric oxide (NO) bioavailability in endothelial cells (ECs). NO bioavailability was examined by Griess's assay, western blot analysis was used to assess protein expression, the level of urea and arginine was evaluated by conventional assay kits. *In vivo* angiogenesis was performed by Matrigel plug assay. In ECs, bromelain increased NO production by increasing intracellular levels of Ca²⁺, activating AMP-activated protein kinase (AMPK), and phosphorylating endothelial nitric oxide synthase (eNOS). Concurrently, bromelain activated the AMPK-regulated autophagy-urea cycle pathway and increased intracellular levels of L-arginine, the precursor of NO, resulting in an increase in NO biosynthesis. Inhibition of bradykinin receptor B₂ (B₂R) or transient receptor potential vanilloid 1 (TRPV1) prevented the activation of Ca²⁺-AMPK-eNOS signaling, autophagy-urea cycle pathway, and NO biosynthesis by bromelain in ECs. Mechanistically, bromelain cleaved kininogen into bradykinin and activated B₂R-TRPV1-Ca²⁺-AMPK-eNOS pathway and autophagy-urea cycle-L-arginine pathway, and these two events may work in concert to promote NO production in ECs. *In vivo* experiments showed that inhibition of B₂R, TRPV1, eNOS, or autophagy activity attenuated bromelain-induced angiogenesis in Matrigel. This study presents novel understanding into the molecular mechanisms underlying the vascular protection of bromelain in the cardiovascular system.

1. Introduction

Bromelain, a proteolytic enzyme, is predominantly found in the stems of pineapples, which is fully absorbed and retains its biological activity in circulation [1]. Stem bromelain has been shown to have anti-inflammatory, antioxidant, and anticoagulant effects and offers protective benefits against conditions like non-alcoholic fatty liver disease (NAFLD) and atherosclerosis [2–5]. Clinically, bromelain is used to treat osteoarthritis, manage inflammation associated with edema, and alleviate postoperative pain and swelling [6,7]. Additionally, bromelain has been suggested to promote neovascularization, increase blood flow, and enhance wound healing by accelerating blood perfusion recovery in

animal models [8–11]. However, its effects and molecular mechanisms on cardiovascular and circulatory health remain unclear.

In circulation, endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) production in endothelial cells (ECs) is essential for maintaining vascular homeostasis. Physiologically, NO is synthesized in response to shear stress, vascular endothelial growth factor (VEGF), and insulin, supporting EC functions by regulating vasodilation and angiogenesis, and inhibiting platelet activation as well as leukocyte adhesion [12]. Bradykinin, an endogenous peptide produced by the action of kallikreins on kininogen within the kinin system, is known to act as a vasodilator by enhancing NO bioavailability through activation of the bradykinin receptor B₂ (B₂R) in ECs [13,14]. Conversely, reduced NO

Abbreviations: AMPK, AMP-activated protein kinase; B2R, bradykinin receptor B2; ECs, endothelial cells; ENOS, endothelial nitric oxide synthase; HMECs, human microvascular endothelial cells; NAFLD, non-alcoholic fatty liver disease; NO, nitric oxide; TRPV1, transient receptor potential vanilloid 1; VEGF, vascular endothelial growth factor; WT, wild type.

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bioavailability due to impaired eNOS activity is closely linked to endothelial dysfunction in cardiovascular diseases, such as hypertension, diabetes, and heart failure [12]. Mechanistically, NO production depends on eNOS activity, which is tightly controlled by Ca^{2+} - and kinase-dependent signaling pathway [12]. This regulation catalyzes the conversion of L-arginine into NO and the co-product L-citrulline in response to endogenous stimuli and medications like statins [12]. Recently, we reported that Ca^{2+} influx via transient receptor potential vanilloid receptor 1 (TRPV1) activates AMP-kinase (AMPK), which phosphorylates eNOS at Ser1177, thereby enhancing NO bioavailability [12,15]. Additionally, studies have shown that autophagy plays a role in NO production induced by shear stress and VEGF-mediated angiogenesis [16,17]. Our recent findings revealed that TRPV1-AMPK signaling is also involved in activating autophagy and increasing intracellular L-arginine levels via the urea cycle, thereby providing substrates for NO synthesis [18]. Nevertheless, the information about interlock regulatory mechanisms between bromelain and eNOS-NO signaling in treating cardiovascular disease and wound healing is limited.

Given the beneficial effects of bromelain on cardiovascular health and wound healing, this study aimed to investigate its impact on NO bioavailability and the underlying molecular mechanisms in ECs. First, we explored the molecular mechanisms through which bromelain affects ECs by catalyzing kininogen, leading to elevated bradykinin levels. Second, we examined how bromelain activates the B₂R-TRPV1-AMPK signaling pathway to stimulate eNOS, thereby enhancing NO bioavailability. Finally, we demonstrated that B₂R-TRPV1-AMPK signaling also mediates bromelain-induced autophagy and the urea cycle, which increases intracellular L-arginine levels. This study presents a novel understanding of the molecular actions of bromelain that support its cardiovascular and circulatory benefits.

2. Materials and methods

2.1. Reagents

Bromelain, acridine orange, baflomycin A1 (BafA1), chloroquine (CQ), icatibant, compound C (C.C.) and SSR240612 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). The recombinant kininogen protein (10529-H08H) was sourced from Sino Biological (Beijing, China). The MTT assay kit, Griess reagent, capsazepine (CPZ), and SB-366791 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Matrigel was provided by Corning, Inc. (Corning, NY, USA). Rabbit antibodies for phospho-AMPK (p-AMPK, #2535), microtubule-associated protein 1 A/1B-light chain 3 (LC3, #4108), and p62 (#5114) were from Cell Signaling Technology (Beverly, MA, USA), while rabbit antibodies against AMPK (A12718), Ser1177-eNOS (AP0515), and eNOS (A1548) were obtained from ABclonal Science (Woburn, MA, USA). The mouse antibody for α -tubulin was purchased from Croyez (Taipei, Taiwan). AMPK siRNA (siAMPK, sc-45312) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Lipofectamine RNAiMAX transfection reagent was from Thermo Fisher Scientific (Waltham, MA, USA). The bradykinin kit (EIA-BRAK) was acquired from RayBiotech (Peachtree Corners, GA, USA), and the rabbit antibody against kininogen (ab175386) as well as colorimetric/fluorometric kits for urea (ab83362) and arginine (ab252892) were from Abcam (Cambridge, MA, UK).

2.2. Animals and experimental procedures

This study complied with the Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and was conducted with approval from the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University (Approval No. 20230098). Male wild-type (WT) C57BL/6 mice were procured from the Laboratory Animal Center at the National Taiwan University College of Medicine (Taipei, Taiwan). The mice were housed in a controlled barrier

environment with a 12-hour light/dark cycle, a stable temperature of 22°C, and a relative humidity of 40–60 %. They were grouped in cages with a maximum of five animals per cage and provided ad libitum access to a standard chow diet. At eight weeks of age, the mice received subcutaneous injections of Matrigel mixed with the designated treatments into their hind legs. After a seven-day treatment period, the mice were euthanized using CO₂ inhalation. The Matrigel plugs were then harvested for further analysis.

2.3. Cell culture

Human microvascular endothelial cells (HMECs) were obtained from the Centers for Disease Control (Atlanta, GA, USA). The cells were maintained in DMEM supplemented with 5 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Additionally, the culture medium included 20 % Endothelial Cell Growth Medium MV2 (Promocell, Heidelberg, Germany), which contained 5 % fetal calf serum, 5 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL insulin-like growth factor (IGF), 0.5 ng/mL vascular endothelial growth factor 165 (VEGF165), 1 µg/mL ascorbic acid, and 0.2 µg/mL hydrocortisone. The human monocytic cell line THP-1 (Bioresource Collection and Research Center, Hsinchu, Taiwan) was cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (HyClone, Logan, UT, USA). HMECs and THP-1 were incubated at 37°C in a humidified atmosphere with 5 % CO₂ to support optimal growth and function.

2.4. Cell viability

Cell viability was assessed using the MTT assay following the manufacturer's protocol. HMECs were treated with the specified experimental conditions for 24 h, after which MTT reagent was added to the culture. Following incubation, the resulting formazan crystals were solubilized, and the absorbance was measured at 570 nm using a microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The absorbance values were used to quantify cell viability relative to the control group.

2.5. Immunocytochemistry

HMECs were seeded into 4-well plates and treated with the indicated conditions for 9 h. Following treatment, the cells were fixed with 4 % paraformaldehyde for 15 min at room temperature (25°C) and permeabilized with 70 % ethanol for 30 min. Cells were blocked with 2 % bovine serum albumin (BSA) to prevent nonspecific binding for 1 h at 25°C. The cells were then incubated with a rabbit anti-LC3 antibody for 2 h at 25°C, followed by conjugation with the appropriate secondary antibody overnight at 4°C. LC3-II puncta were visualized under a Leica DMIRB fluorescence microscope (Leica, Wetzlar, Germany) equipped with LAS V4.12 software. The number of LC3-II puncta was quantified from the captured photomicrographs.

2.6. Western blot analysis

HMECs were lysed using immunoprecipitation lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 300 mmol/L NaCl, 1 % Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL leupeptin, and 10 µg/mL aprotinin. The lysates were clarified by centrifugation, and protein concentrations were determined. Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8–10 % gels. After electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5 % skim milk in Tris-buffered saline with 0.1 % Tween-20 (TBST) for 1 h at room temperature (25°C). Subsequently, the

membranes were incubated overnight at 4°C with the specified primary antibodies, followed by a 2-h incubation at room temperature (25°C) with the appropriate horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit (PerkinElmer, Waltham, MA, USA). Band intensity was quantified using TotalLab 1D analysis software (TotalLab, Newcastle Upon Tyne, UK).

2.7. Determination of nitrite production

NO is metabolized into nitrates and nitrates, with nitrite levels as an indicator of NO production in HMECs. Following the specified treatments, cell culture supernatants were collected and mixed with an equal volume of Griess reagent. The mixture was incubated at room temperature for 15 min to allow color development. The absorbance of the samples was measured at 540 nm using a microplate spectrophotometer. Sodium nitrite was used as a standard to quantify nitrite concentrations in the culture medium, indirectly measuring NO production.

2.8. Acidic vesicular organelles

Acridine orange staining was employed to evaluate the formation of autophagolysosomes by detecting acidic vesicular organelles. Stained cells were imaged using a Nikon TE2000-U fluorescence microscope equipped with a digital imaging system (QCapture Pro 6.0, QImaging, Surrey, BC, Canada). The number of acidic vesicular organelles in captured photomicrographs was quantified to assess autophagolysosome formation.

2.9. Measurement of urea cycle intermediates

The intracellular concentrations of urea and arginine were quantified using commercially available assay kits, adhering strictly to the manufacturer's instructions. Absorbance readings at 450 nm were obtained using a microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to determine the concentrations. To evaluate the effects of the treatments, the concentration differences (Δ) between the treatment groups and the vehicle control group were calculated.

2.10. Small interfering RNA transfection

HMECs were seeded into 3.5 cm culture dishes and subjected to serum starvation for 6 h to synchronize cellular activity. After starvation, the cells were incubated for 24 h in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA) containing either scrambled siRNA or AMPK-specific siRNA, delivered using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). Following transfection, the medium was replaced with a fresh, complete culture medium, and the cells were prepared for subsequent experimental procedures.

2.11. Matrigel plug angiogenesis in mice

Matrigel was prepared by mixing with 50 U/mL heparin and the designated treatments. The mixture was subcutaneously injected into the flanks of the mice. After 7 days, the mice were euthanized using CO₂, and the Matrigel plugs were isolated. The plugs were photographed to document gross morphological changes. As an indicator of angiogenesis, hemoglobin content was extracted from the plugs using 0.04 % ammonium hydroxide solution. The hemoglobin levels were quantified by measuring absorbance at 416 nm using a spectrophotometer.

2.12. In vitro mononuclear-endothelial cell adhesion assay

HMECs were seeded into 3.5 cm culture dishes and treated with bromelain (2 µg/mL) for 6 h, followed by TNFα (10 ng/mL) for an

additional 12 h. BCECF-AM fluorescent-labeled THP-1 cells (1 × 10⁵ cells/mL) were added to the culture medium of HMECs or BAECs and incubated for 1 h. Photomicrographs were digitally captured using a Leica DMIRB fluorescence microscope (Leica, Wetzlar, Germany) equipped with LAS V4.12 software. The cell number of monocyte adherence to ECs was quantified from the captured photomicrographs.

2.13. Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM), derived from five independent experiments. After verification, our data was not normally distributed. Comparisons between two groups were performed using the Mann-Whitney *U* test. For comparisons involving more than two groups, the Kruskal-Wallis test was used, followed by Dunn's post-hoc test for pairwise comparisons. All statistical analyses were conducted using SPSS software version 8.0 (SPSS Inc., Chicago, IL, USA). A *p* < 0.05 was considered statistically significant.

3. Results

3.1. Bromelain-increased NO production is mediated by eNOS activity

To determine the optimal concentration of bromelain for inducing NO production, HMECs were treated with various concentrations of bromelain (0.125–8 µg/mL). The data demonstrated that the concentrations of bromelain from 0.125 to 2 µg/mL for 24 h without changing cell viability (Fig. 1A and B) but significantly upregulated NO production (Fig. 1C). Moreover, treatment with 2 µg/mL of bromelain enhanced NO production in a time-dependent manner (Fig. 1D). Despite the increase in NO production, western blot analysis indicated that bromelain treatment did not alter the protein levels of eNOS (Fig. 1E). Furthermore, the increase in NO bioavailability induced by bromelain was entirely blocked by pretreating with the NOS inhibitor L-NAME (Fig. 1F), suggesting that bromelain stimulates NO production through the activation of eNOS activity.

3.2. Bromelain exhibits enzymatic activity on kininogen and releases bradykinin

To examine the effect of bromelain on kininogen, recombinant kininogen protein (10 ng) was incubated with bromelain (2 µg/mL) at 37°C. Western blot analysis revealed that bromelain induced time-dependent degradation of kininogen into smaller fragments. In contrast, when bromelain was heated to boiling temperatures, its enzymatic activity on kininogen was abolished entirely (Fig. 2A). Similarly, in HMEC lysates, the protein levels of kininogen decreased following incubation with bromelain at 37°C (Fig. 2B). Additionally, bromelain treatment significantly increased bradykinin levels in the culture medium, with peak levels observed at 10 min, and these elevated bradykinin levels remained sustained for up to 90 min (Fig. 2C). These findings suggest that bromelain exerts enzymatic activity on kininogen, promoting the release of bradykinin, which may play a role in mediating its biological effects on ECs.

3.3. The B₂R-TRPV1-Ca²⁺ signaling pathway is vital in NO production by bromelain

To determine whether Ca²⁺ is mediated bromelain-induced NO production, ECs were incubated with 2 µg/mL bromelain. Compared to the vehicle-treated group, the intracellular Ca²⁺ levels were increased in a time-dependent manner, peaking at 15 min and gradually returning to baseline by 240 min (Fig. 3A). Pretreatment of HMECs with the Ca²⁺ chelators EDTA or EGTA inhibited the bromelain-induced increase in both intracellular Ca²⁺ levels and NO production (Fig. 3B and C). Next, we explored whether the bromelain-provoked increase in intracellular Ca²⁺ and NO biosynthesis is mediated by B₂R in ECs. The results showed

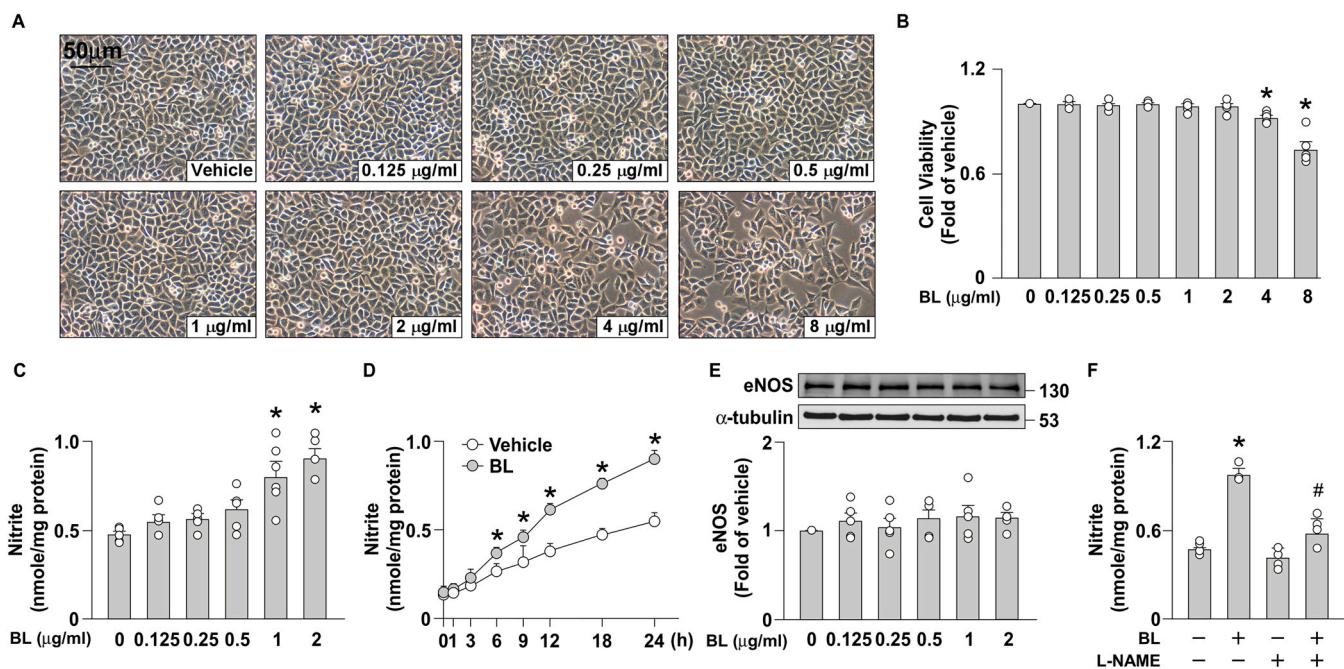


Fig. 1. Bromelain induces NO bioavailability in ECs. HMECs were treated with bromelain (BL) in dose- or time-dependent manner for further experiments. (A and B) Cell viability. (C and D) Nitrite levels. (E) The eNOS protein expression analyzed by western blot. (F) Griess assay results for nitrite levels. Data are expressed as the mean \pm standard error of the mean (SEM), derived from five independent experiments. * p < 0.05 vs. the vehicle-treated group; # p < 0.05 vs. the bromelain-treated group.

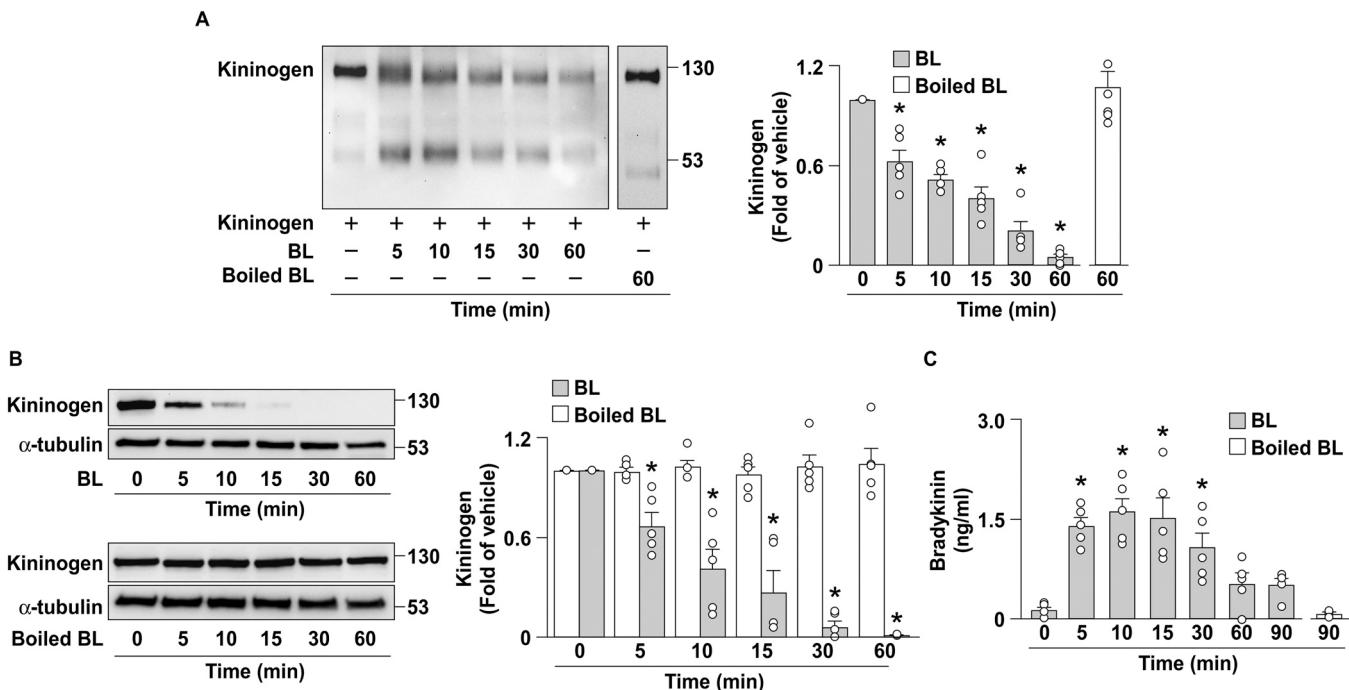


Fig. 2. The enzymatic effect of bromelain on kininogen to increase bradykinin production. Recombinant kininogen protein (A) or HMECs cell lysate (B) was incubated with bromelain (BL, 2 μ g/mL) or boiled bromelain in a time-dependent manner. (A and B) The protein level of kininogen was analyzed by western blot. (C) Measurement of bradykinin levels in the culture medium of HMECs treated with bromelain or boiled bromelain for the indicated times. Data are expressed as the mean \pm standard error of the mean (SEM), derived from five independent experiments. * p < 0.05 vs. the vehicle-treated group.

that the rise in intracellular Ca^{2+} and NO production was abolished by the B_2R inhibitor icatibant (Fig. 3D and E). Additionally, since bradykinin is known to promote Ca^{2+} influx through the $\text{B}_2\text{R}-\text{TRPV1}$ signaling pathway [19], we investigated the participation of TRPV1 in bromelain-conferred beneficial effects. HMECs pretreated with TRPV1

inhibitors, capsazepine (CPZ), and SB366791 inhibited bromelain-evoked increases in intracellular Ca^{2+} levels and NO production (Fig. 3F and G).

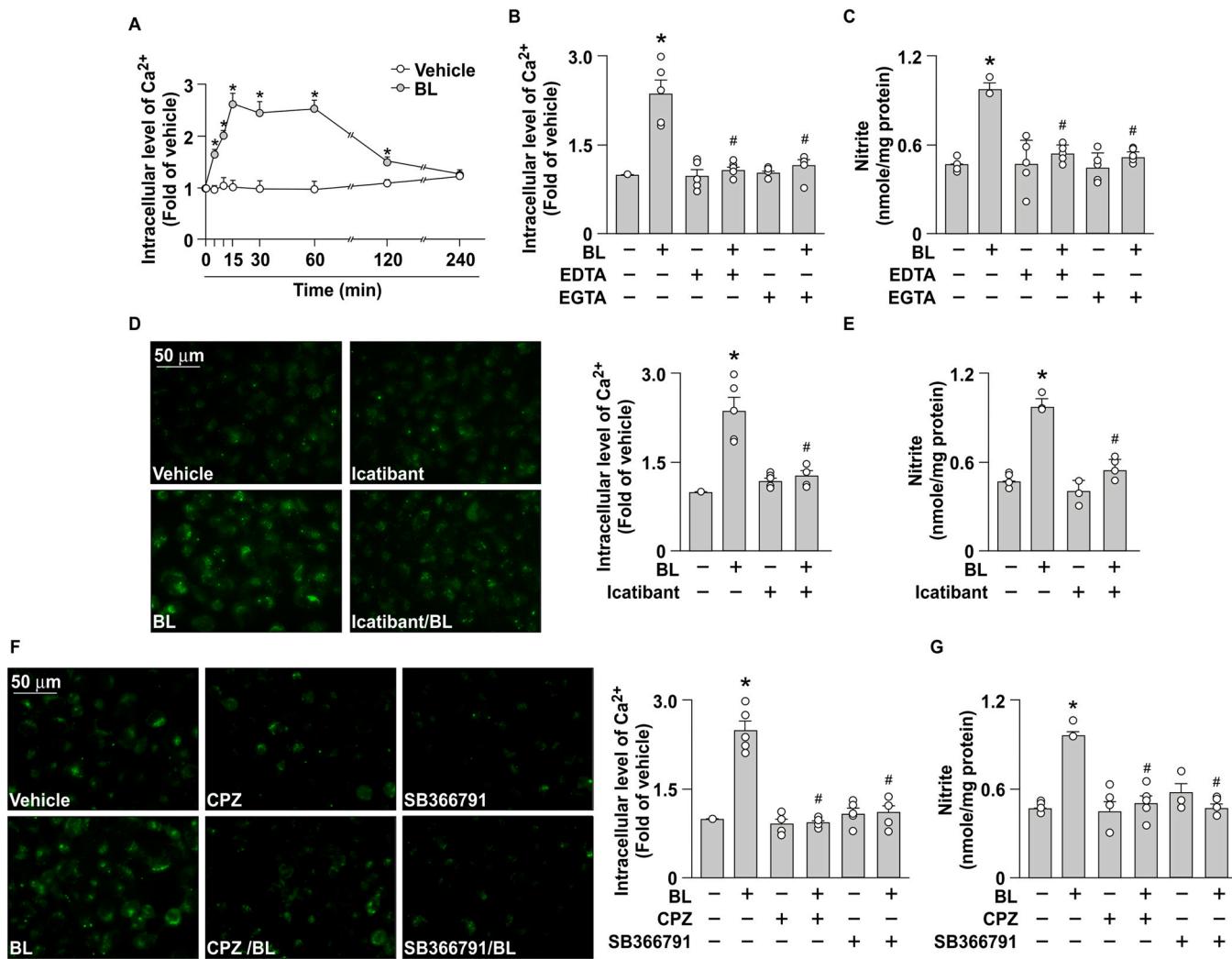


Fig. 3. B₂R-TRPV1-mediated increase in intracellular levels of Ca²⁺ is vital for bromelain-induced NO production. (A) Intracellular Ca²⁺ levels in HMECs treated with bromelain (BL, 2 µg/mL) for the indicated times. (B) Intracellular Ca²⁺ levels in HMECs were incubated with Ca²⁺ chelators, EGTA (0.5 µM), and EDTA (0.5 µM) for 2 h, followed by bromelain treatment for 15 min. (C) Nitrite levels in the culture medium of HMECs were pretreated with Ca²⁺ chelators for 2 h, following by bromelain for 18 h were measured. (D) Representative fluorescence images and intracellular Ca²⁺ levels in HMECs pretreated with the B₂R inhibitor, icatibant (100 nM), followed by bromelain treatment for 15 min. (E) Nitrite levels in the culture medium of HMECs pretreated with icatibant, assessed by Griess' assay. (F) Representative fluorescence images and intracellular Ca²⁺ levels in HMECs pretreated with TRPV1 antagonists, CPZ (10 µM) or SB366791 (10 µM), followed by bromelain treatment for 15 min. (G) Nitrite levels of HMECs pretreated with TRPV1 antagonists, assessed by Griess' assay. Intracellular Ca²⁺ levels were analyzed using the Fluo-8 calcium assay. Data are expressed as the mean ± standard error of the mean (SEM), derived from five independent experiments. *p < 0.05 vs. the vehicle-treated group; #p < 0.05 vs. the bromelain-treated group.

3.4. The role of the B₂R-TRPV1-Ca²⁺-AMPK pathway in bromelain-caused eNOS activation and NO biosynthesis

The TRPV1-AMPK pathway is widely recognized for its role in regulating NO bioavailability in ECs [15,18]. We wanted to explore the involvement of the B₂R-TRPV1-Ca²⁺ axis in bromelain-increased eNOS activity and NO biosynthesis. The data demonstrate that treatment with bromelain led to a time-dependent increase in the phosphorylation of eNOS at Ser1177 and AMPK (Fig. 4A). To assess the contribution of B₂R and TRPV1 in this process, HMECs were pretreated with specific inhibitors: icatibant for B₂R, and CPZ and SB266791 for TRPV1 antagonism. As shown in Fig. 4B and C, both B₂R and TRPV1 inhibitors effectively blocked the bromelain-induced phosphorylation of eNOS at Ser1177 and AMPK. Furthermore, inhibiting AMPK activity with Compound C (C.C.) completely prevented the bromelain-induced phosphorylation of eNOS at Ser1177 in HMECs (Fig. 4D). These findings confirm that the B₂R-TRPV1-Ca²⁺-AMPK pathway plays a critical role in mediating bromelain-activated eNOS-NO pathway.

3.5. Autophagy is pivotal in bromelain-induced NO production

To explore whether autophagy contributes to bromelain-elicited NO biosynthesis, HMECs were treated with bromelain for varying durations. Our results revealed a time-dependent increase in the levels of LC3-II, with peak expression occurring at 9 h post-treatment. Conversely, the levels of p62 protein declined over time (Fig. 5A). Moreover, the treatment of heat-inactivated bromelain abolished the activation of autophagy in a time-dependent manner (Fig. S1). Acridine orange staining further showed an increase in intracellular acidic vacuoles, reaching a maximum at 9 h, suggesting enhanced autolysosomal activity following bromelain exposure (Fig. 5B). Immunocytochemical analysis confirmed that bromelain treatment promoted autophagosome formation, as evidenced by a higher number of LC3 puncta (Fig. 5C). To determine the role of autophagy in bromelain-increased NO generation, HMECs were pretreated with chloroquine (CQ) or bafilomycin A1 (BafA1), both of which inhibit autophagy. As shown in Fig. 5D and E, treatment with CQ or BafA1 inhibited both the autophagic flux and the bromelain-induced

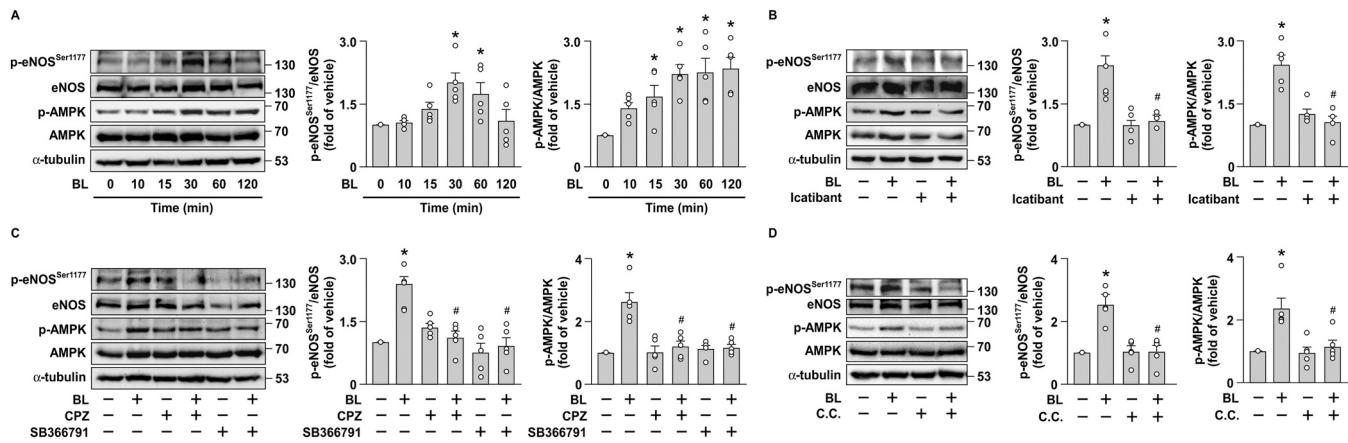


Fig. 4. Bromelain induces the eNOS phosphorylation at Ser1177 via the B₂R-TRPV1-AMPK signaling pathway. (A) Western blot analysis of phosphorylated eNOS, total eNOS, phosphorylated AMPK, and total AMPK in HMECs treated with bromelain (BL, 2 µg/mL) for indicated times (0, 10, 15, 30, 60, 120 min). Western blot analysis of phosphorylated eNOS, total eNOS, phosphorylated AMPK, and total AMPK in HMECs pretreated with (B) capsazepine (CPZ, 10 µM) or SB366791 (10 µM), (C) icatibant (100 nM) and (D) compound C (C.C., 10 µM) for 2 h, and then treated with bromelain for 30 min. Data are expressed as the mean ± standard error of the mean (SEM), derived from five independent experiments. *p < 0.05 vs. the vehicle-treated group; #p < 0.05 vs. the bromelain-treated group.

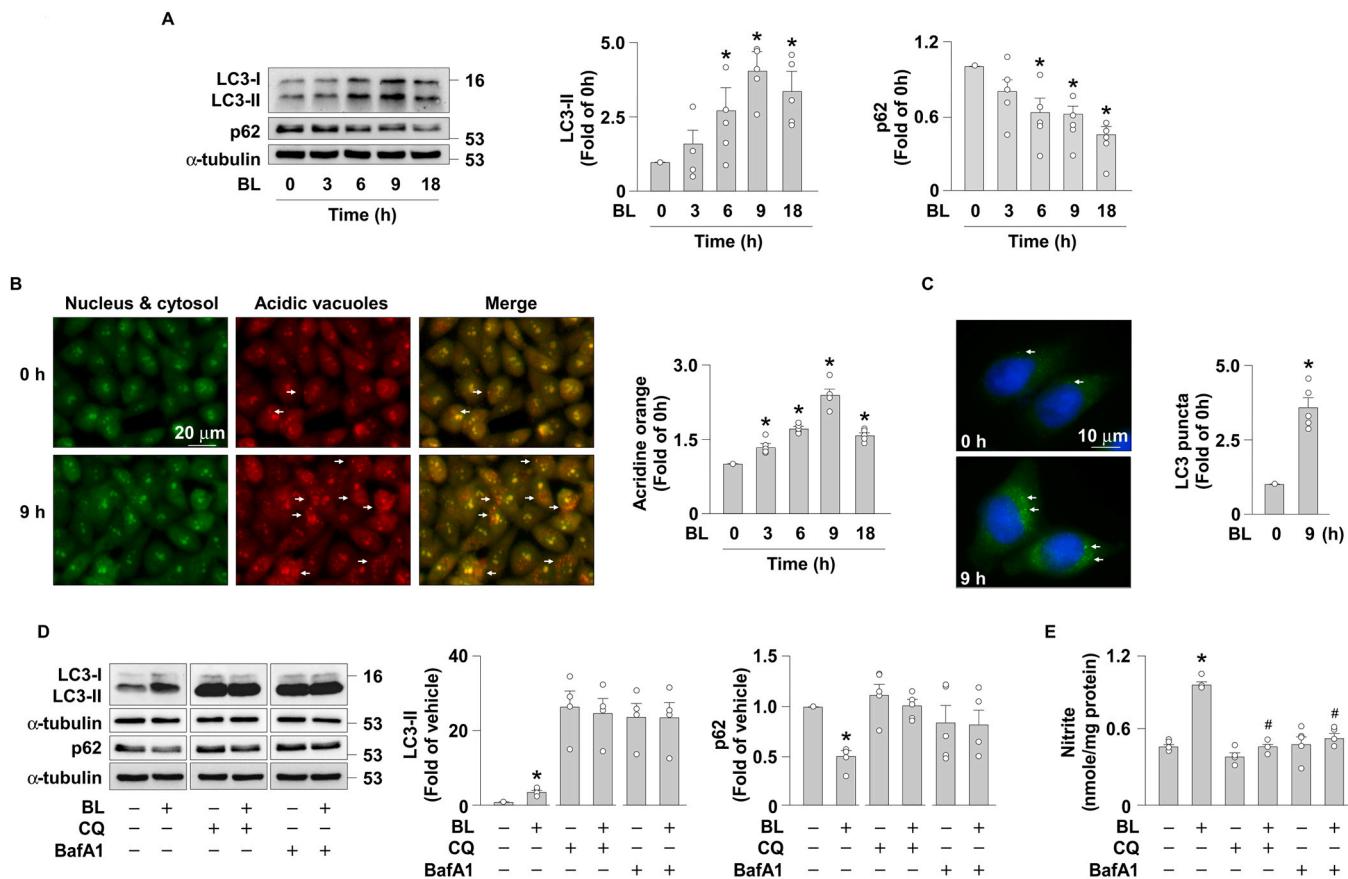


Fig. 5. Bromelain promotes NO production by activating autophagy. HMECs were incubated with bromelain (BL, 2 µg/mL) in time-dependent manner. (A) The representative images and quantitative results of the protein levels of LC3, p62, and α-tubulin. (B) Acridine orange staining. (C) LC3 puncta. Arrowheads indicate (B) acidic vacuoles or (C) LC3 puncta. HMECs were pretreated with autophagy inhibitors, chloroquine (CQ, 40 µM), and bafilomycin A (BafA1, 10 nM) for 2 h, then cultured with bromelain for another 9 h or 18 h. (D) The protein level of LC3 at 9 h and p62 at 18 h. (E) Nitrite levels of HMECs for indicated treatment. Data are expressed as the mean ± standard error of the mean (SEM), derived from five independent experiments. *p < 0.05 vs. the vehicle-treated group, CQ-alone group, or BafA1-alone group; #p < 0.05 vs. the bromelain-treated group.

increase in NO production. These results suggest that autophagy activation is a crucial mechanism for bromelain-increased NO biosynthesis in ECs.

3.6. The B₂R-TRPV1-AMPK signaling is essential for bromelain-triggered autophagy activation

We have previously shown that the TRPV1-AMPK-autophagy-urea

cycle-L-arginine signaling is pivotal for NO biosynthesis in ECs [18]. Here, we aimed to investigate whether the B₂R-TRPV1-AMPK signaling axis also plays a role in bromelain-induced autophagy flux. Pretreatment with the B₂R inhibitor, icatibant, completely blocked the bromelain-induced autophagic flux and inhibited LC3 puncta formation (Fig. 6A-C). Moreover, icatibant pretreatment also prevented the bromelain-induced increases in intracellular L-arginine and urea levels, suggesting that B₂R is essential for activating the autophagy-urea cycle-L-arginine signaling in response to bromelain (Fig. 6D and E). In a similar manner, inhibiting TRPV1 activity using specific antagonists reduced both autophagy activation and LC3 puncta formation following bromelain treatment (Fig. 7A and B). Additionally, the bromelain-induced increases in L-arginine and urea levels were attenuated in HMECs pretreated with TRPV1 inhibitors (Fig. 7C). To further investigate the role of AMPK in this process, we employed AMPK siRNA and the AMPK inhibitor Compound C (C.C.). As shown in Fig. 7D and E, AMPK knockdown via siRNA significantly impaired bromelain-induced autophagy activation and LC3 puncta formation. Furthermore, inhibition of AMPK activity with C.C. abolished bromelain-induced NO production in HMECs (Fig. 7F). In addition, AMPK inhibition also inhibited bromelain-induced autophagy activation and LC3 puncta formation (Fig. 7G and H). These findings suggest that the B₂R-TRPV1-AMPK signaling is critical in regulating autophagy and subsequent NO biosynthesis in ECs in response to bromelain treatment.

3.7. Bromelain-induced NO bioavailability and angiogenesis *in vivo*

To investigate the molecular mechanisms underlying bromelain-induced NO bioavailability and its effects on angiogenesis in ECs, the *in vivo* Matrigel plug assay was performed. C57BL/6 WT mice were subcutaneously injected with Matrigel plugs containing various doses of bromelain, with Matrigel plugs containing simvastatin as a positive control. As shown in Fig. 8A, bromelain dose-dependently increased vascularization in the Matrigel plugs of WT mice. By contrast, Matrigel

plugs containing antagonists to inhibit the activation of B₂R, TRPV1, eNOS, AMPK, or autophagy significantly reduced bromelain-increased angiogenesis in WT mice (Fig. 8B). Together, these results demonstrate that the B₂R-TRPV1-AMPK signaling pathway, which activates eNOS activity and promotes autophagy flux, plays a critical role in the beneficial effects of bromelain on ECs and angiogenesis.

4. Discussion

Bromelain is a proteolytic enzyme with multiple beneficial effects on the human body. It aids digestion and nutrient absorption in the gastrointestinal tract through its proteolytic activity [20]. Clinically, bromelain treats osteoarthritis and inflammation-associated edema, such as that caused by traumatic injury or postoperative recovery. It exerts its anti-inflammatory effects by reducing the levels of prostaglandin E2, cyclooxygenase, and inflammatory cytokines like interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α [20]. Additionally, bromelain has been proposed as a treatment for coagulation disorders due to its anti-coagulant properties. Our previous work, along with that of others, has shown that bromelain decreases the levels of fibrinogen and factor XIIIa, increases anti-coagulation proteins, and enhances fibrinolysis by elevating plasminogen levels, which helps prevent clot formation in NAFLD mice [4,21]. Despite these recognized cardiovascular benefits, the molecular mechanisms through which bromelain affects the vascular system remain incompletely understood. In this study, we identified the potential enzymatic action of bromelain on proteins in ECs, activating downstream signaling pathways involved in NO bioavailability, which is crucial for vascular health. We show that bromelain acts as an enzyme in the kinin system, cleaving kininogen into bradykinin, a known vasodilator in circulation. Additionally, we demonstrate that bromelain activates eNOS and increases NO biosynthesis through the B₂R-TRPV1-AMPK pathway. Furthermore, as previous findings, the autophagy-urea cycle-L-arginine signaling is essential for regulating NO bioavailability in ECs [18]. Our findings reveal that

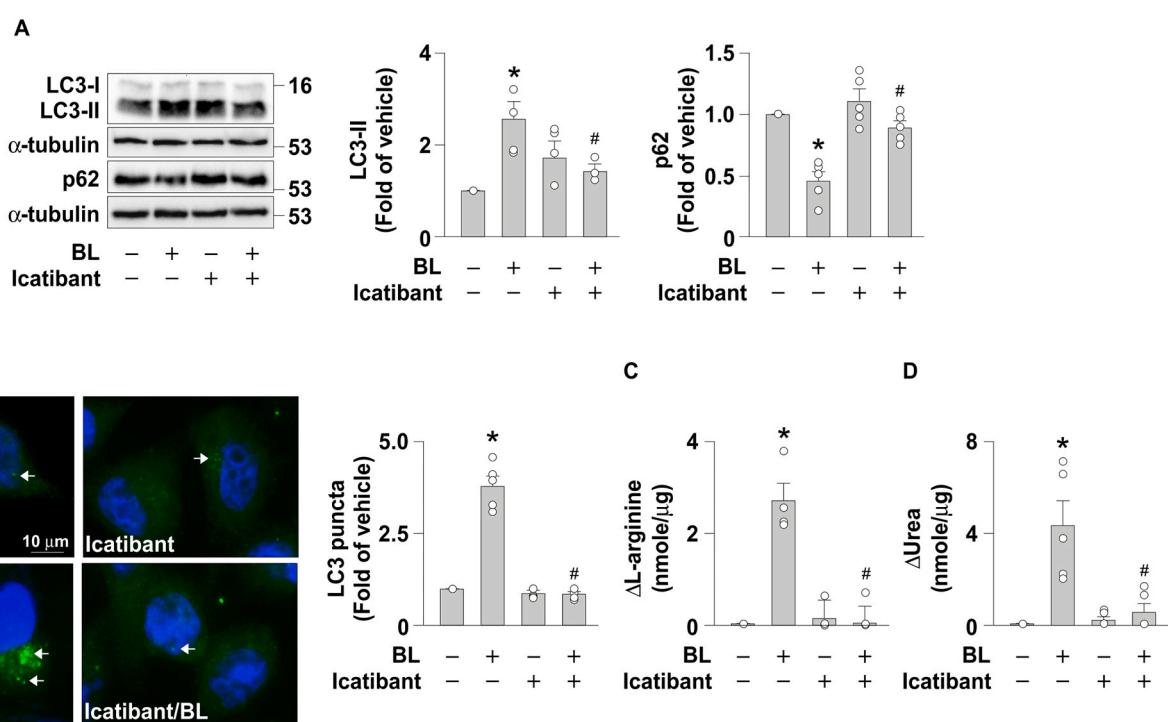


Fig. 6. The B₂R signaling mediates bromelain-induced autophagy-urea cycle pathway and NO biosynthesis. HMECs were incubated with the B₂R inhibitor, icatibant (100 nM), for 2 h, followed by incubation with bromelain (BL, 2 μ g/mL) for an additional 9 h or 18 h. (A) The protein level of LC3 at 9 h and p62 at 18 h. (B) LC3 puncta in HMECs. Arrowheads indicate LC3 puncta. (C and D) Changes in the levels (Δ) of L-arginine and urea. Data are expressed as the mean \pm standard error of the mean (SEM), derived from five independent experiments. * p < 0.05 vs. the vehicle-treated group; # p < 0.05 vs. the bromelain-treated group.

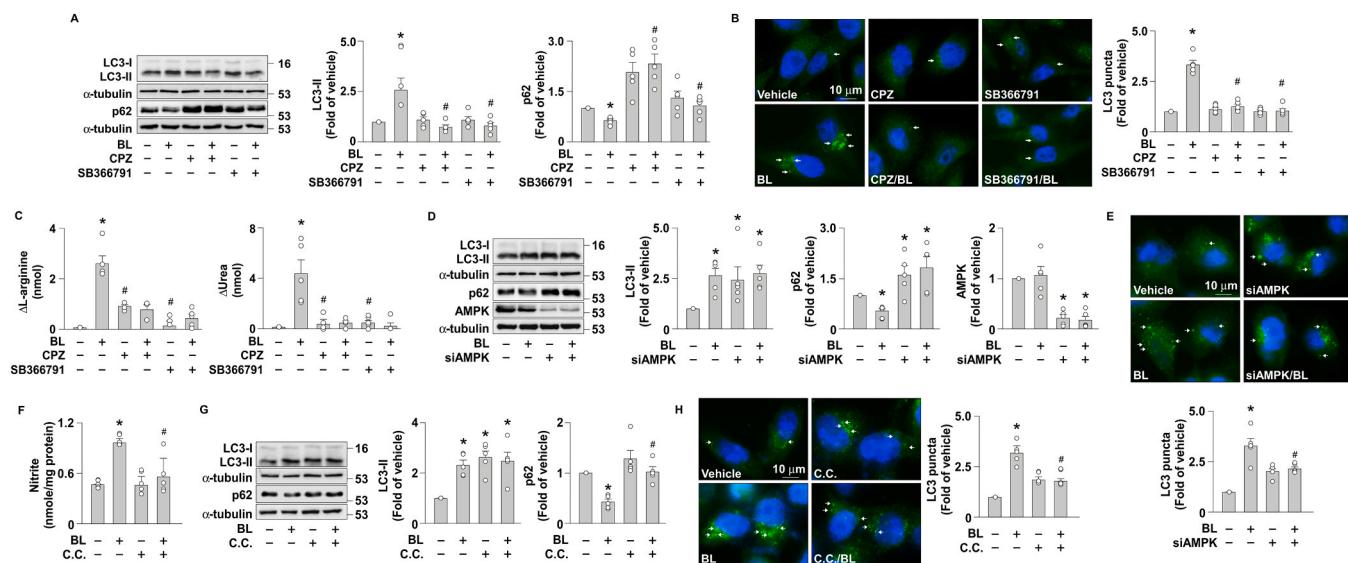


Fig. 7. TRPV1-AMPK pathway is required for bromelain-induced autophagy-urea cycle and NO biosynthesis. HMECs were incubated with TRPV1 antagonists, capsazepine (CPZ, 10 μ M) or SB366791 (10 μ M) for 2 h, followed by bromelain (BL, 2 μ g/mL) for another 9 h or 18 h. (A) The protein level of LC3 at 9 h, and p62 at 18 h. (B) LC3 puncta in HMECs. Arrowheads indicate LC3 puncta. (C) The changes in the levels (A) of L-arginine and urea. HMECs were transfected with AMPK siRNA (100 nM) for 24 h or compound C (C.C., 10 μ M) for 2 h, following by bromelain for another 9 h or 18 h. (D) The protein level of LC3 at 9 h, and p62 and AMPK at 18 h. (E) LC3 puncta in HMECs. Arrowheads indicate LC3 puncta. (F) Nitrite levels of HMECs for indicated treatment. (G) The protein of LC3 at 9 h, and p62 at 18 h. (H) LC3 puncta in HMECs. Arrowheads indicate LC3 puncta. Data are expressed as the mean \pm standard error of the mean (SEM), derived from five independent experiments. * p < 0.05 vs. the vehicle-treated group; # p < 0.05 vs. the bromelain-treated group.

the B₂R-TRPV1-AMPK pathway is critical in bromelain-activated autophagy flux and L-arginine biosynthesis. Thus, the enzymatic activity of bromelain on kininogen leads to the release of bradykinin, which promotes NO bioavailability and angiogenesis via the B₂R-TRPV1-AMPK-eNOS and autophagy-urea cycle-L-arginine signaling pathways (Fig. 8C). Collectively, our study delivers novel viewpoints into the biological actions and regulatory mechanisms of bromelain in NO production and endothelial cell function, highlighting its potential as a therapeutic strategy for cardiovascular diseases.

Stem bromelain, a proteolytic enzyme extracted from pineapple with a molecular weight of 23 kDa, cannot directly pass through the cellular membrane [22]. Despite this, the mechanisms by which bromelain activates receptor-mediated signaling pathways and its cellular effects remain unclear. The kinin system, which consists of the substrate kininogen, the enzyme kallikrein, and the end product bradykinin, plays a key role in various biological processes. Bradykinin is generated through the enzymatic action of kallikrein on high molecular weight kininogen and exerts its effects through two specific receptors, B₁R and B₂R [23]. Reports by Feng et al. and Madeddu et al. have shown that B₂R deficiency increases blood pressure and heightens the risk of age-related cardiac dysfunction [24,25]. Additionally, B₂R activation has been reported to reduce renal fibrosis in animal models of unilateral ureteral obstruction [26]. The data revealed that the kinin system, particularly bradykinin signaling via B₂R, plays a crucial role in the pathophysiology of cardiovascular diseases [23,27–29]. However, bradykinin is rapidly degraded in the plasma by angiotensin-converting enzyme (ACE) and other kininases [28,30]. ACE inhibition, which prevents bradykinin degradation, has become a therapeutic target in the treatment of hypertension and offers cardioprotective effects in ischemic heart disease [31,32]. Beyond inhibiting bradykinin degradation, increasing bradykinin levels in circulation is another way to enhance the cardioprotective effects of the kinin system. Our results revealed that bromelain acts on kininogen, increasing bradykinin levels in the culture medium within 5–30 min of treatment, gradually decreasing by 90 min. This finding contrasts with the results of Kumakura et al., who suggested that bromelain increases bradykinin-degrading activity, thus reducing bradykinin levels in the serum of rats after bromelain injection [33].

This observation aligns with our findings, which show a decrease in bradykinin levels in the medium after 30 min of bromelain treatment.

Kallikrein, a mediator between the kinin system and the fibrinolysis pathway, is activated from prekallikrein by factor XII in the intrinsic coagulation pathway. It then converts plasminogen into plasmin to promote fibrin degradation [34]. Kumakura et al. demonstrated that bromelain decreases prekallikrein levels in rat [33], which is consistent with our observation of increased kallikrein levels in HMECs treated with bromelain (data not shown). This suggests that bromelain may promote the conversion of prekallikrein to kallikrein, enhancing fibrinolytic activity. Together with our previous study [4], we demonstrated that bromelain exerts anticoagulant effects by promoting fibrinolytic activity, alleviating the deregulated blood coagulation in the NAFLD animal model. We hypothesize that the effects of bromelain on fibrinolysis and bradykinin levels contribute to its beneficial effects on EC function and vascular health. Additionally, endothelial dysfunction also plays a crucial role in the pathological progression of atherosclerosis. Our previous study has revealed that bromelain ameliorates atherosclerosis by activating autophagy and antioxidant pathways in the aorta and liver of *apoε*^{-/-} mice. Particularly, bromelain improves endothelial function by activating autophagy flux in *apoε*^{-/-} mice, as evidenced by the increased expression of LC3 in the endothelium of the aorta from bromelain-treated *apoε*^{-/-} mice immunostaining [3]. Furthermore, under the pathological condition with TNFα treatment, our data showed that bromelain prevented TNFα-induced NO reduction and monocyte adherence on ECs (Fig. S3). In summary, these results indicated that the cardioprotective benefits of bromelain are linked to the kinin system, where bromelain increases bradykinin levels and activates downstream signaling pathways that promote vascular health.

Bradykinin is known to increase vascular permeability and NO bioavailability, which in turn enhances EC functions [28]. There are two types of bradykinin receptors on the cell surface: B₂R, constitutively expressed under normal physiological conditions, and B₁R, induced in response to inflammation and tissue injury [23]. Studies have shown that B₂R antagonist-treated or B₂R knockout mice exhibit impaired NO release due to dysfunction in the kinin system and elevated blood pressure, indicating that B₂R plays a critical role in maintaining vascular

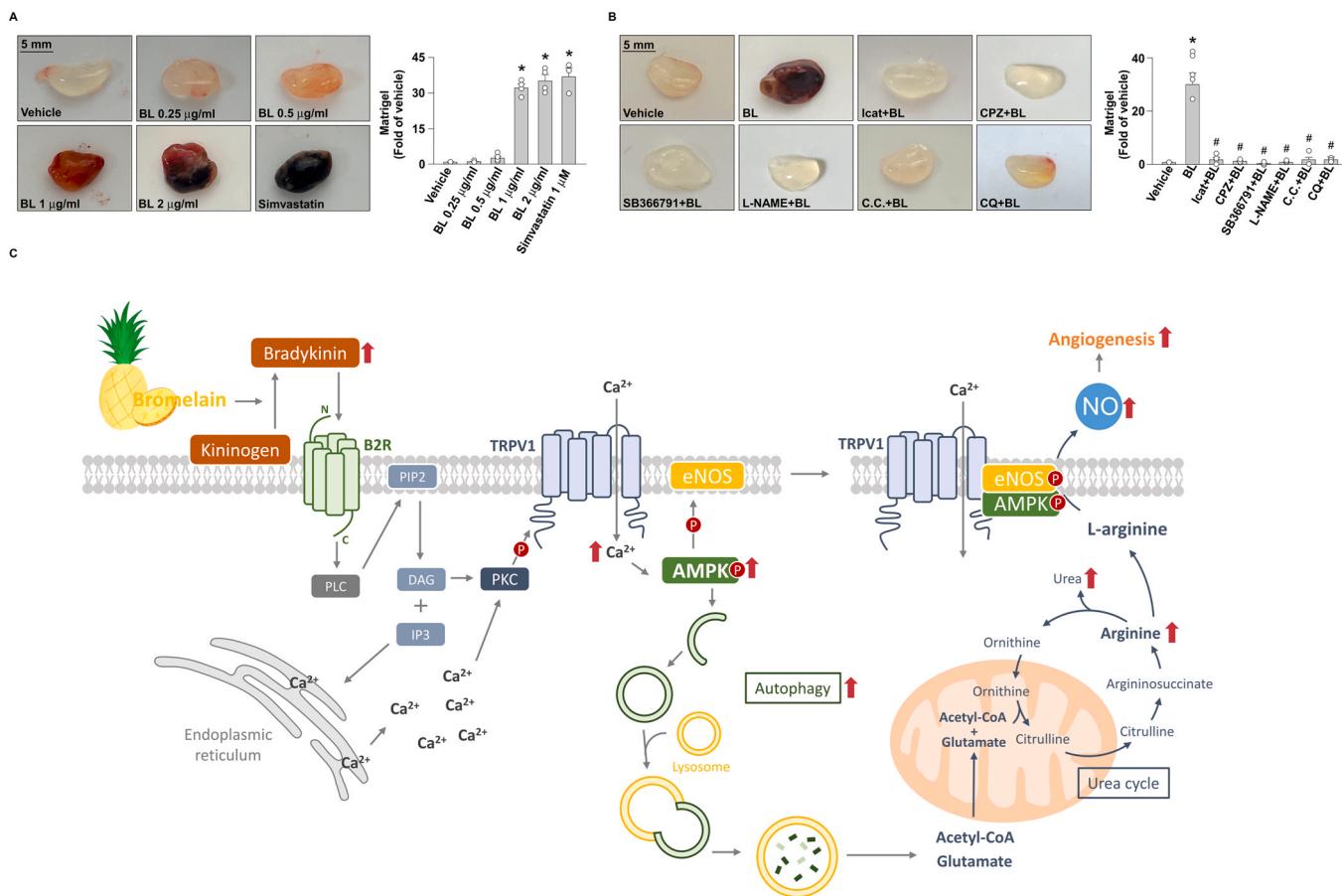


Fig. 8. Bromelain promotes angiogenesis via the B₂R-TRPV1-eNOS-AMPK-autophagy pathway. Eight-week-old mice were subcutaneously injected with Matrigel plugs with indicated dose of bromelain (BL, 0.25, 0.5, 1, 2 µg/mL) or simvastatin (1 µM) as positive control, or bromelain (2 µg/mL) with various inhibitors, including icatibant (100 nM), capsazepine (CPZ, 10 µM), SB366791 (10 µM), L-NAME (400 nM), compound C (10 µM) and chloroquine (CQ, 40 µM). On day 7 post-administration, plugs were removed and photographed, and the dissolved hemoglobin content was extracted by 0.04 % NH₃. (A and B) The representative images and quantitative results of *in vivo* Matrigel plugs assay. Data are expressed as the mean ± standard error of the mean (SEM), derived from five independent experiments. *p < 0.05 vs. the vehicle-treated group; #p < 0.05 vs. the bromelain-treated group. (C) Schematic representation of the proposed molecular mechanisms by which bromelain stimulates the B₂R-TRPV1-AMPK signaling cascade and induces autophagy-urea cycle activation to promote L-arginine biosynthesis and enhance NO bioavailability in ECs.

homeostasis [25,35–37]. Our study found that bromelain-triggered NO biosynthesis was blocked by a B₂R inhibitor. In contrast, pretreatment with a B₁R inhibitor SSR240612 did not have the same effect (data not shown). This suggests that B₂R activation is necessary for bromelain-induced NO production. Activation of B₂R triggers a second messenger pathway involving phospholipase C, which stimulates the release of Ca²⁺ from the endoplasmic reticulum and activates protein kinase C (PKC) [38]. The increased intracellular Ca²⁺ further activates PKC, which phosphorylates TRPV1, leading to enhanced Ca²⁺ influx [19]. It has been demonstrated that the TRPV1-Ca²⁺-AMPK pathway is essential for NO production [15]. Our findings indicated that bromelain increased intracellular Ca²⁺ levels via the activation of both B₂R and TRPV1, subsequently activating AMPK and phosphorylating eNOS, ultimately promoting NO production. We have previously shown that autophagy plays a key role in NO production, as it increases the *de novo* biosynthesis of L-arginine by regulating urea cycle [18]. This study demonstrated that the B₂R-TRPV1-AMPK signaling pathway mediates bromelain-induced autophagy flux activation, contributing to NO production. Specifically, the activation of eNOS increases its enzymatic activity, at the same time, the induction of autophagy flux enhances intracellular L-arginine levels, both of which are crucial for bromelain-induced NO production in ECs.

Interestingly, several studies have suggested that bromelain accelerates wound healing through its anti-inflammatory and anti-

coagulation effects, as well as its ability to remove the necrotic layer from the dermis [21,39,40]. Additionally, bromelain has been proposed to enhance wound healing by promoting angiogenesis [9,11,41,42]. During the proliferative phase of wound healing, angiogenesis is critical in supplying oxygen and nutrients to the wound site [41]. In addition to reducing inflammatory cytokines, previous studies have shown that bromelain improves wound healing by stimulating neovascular formation and increasing partial oxygen pressure, thereby enhancing blood perfusion to the affected area [9,11,42]. While bromelain-induced angiogenesis has been well-documented in animal models, the molecular mechanisms underlying this effect are still unclear. In this study, we utilized an *in vivo* Matrigel assay to investigate the molecular mechanisms of bromelain in angiogenesis. Our results suggest that the B₂R-TRPV1-AMPK-autophagy signaling is involved in bromelain-elicited angiogenesis.

Notably, the wound-healing process in diabetes patients is hindered by risk factors such as excessive inflammatory response, oxidative stress, and reduced NO production in microvascular vessels [43]. In addition to managing blood glucose, enhancing NO production is a key therapeutic approach to improve healing rates in diabetes patients, owing to NO's anti-inflammatory, antioxidant, and angiogenesis-promoting properties [44]. For example, Fetterman et al. demonstrated that autophagy activation can enhance NO signaling in individuals with diabetes [45]. More recently, bromelain has been suggested to lower fasting blood glucose in

type 1 diabetic animal model [46]. Lu et al. showed that bromelain suppresses pro-inflammatory cytokines triggered by advanced glycation end-products and enhances wound healing in an in vitro diabetes model [47]. Fathi et al. also reported that bromelain accelerates wound healing in diabetic animal models by promoting neovascularization and reducing inflammation [9,40]. Our findings support these results, indicating that bromelain may aid wound healing in diabetes by activating autophagy and increasing NO bioavailability in ECs. These studies suggest that bromelain may help regulate blood glucose levels in individuals with diabetes. However, further investigation is needed to clarify its beneficial effects and underlying mechanisms on glucose homeostasis.

Regarding the specificity of pharmacological inhibitors, including CPZ, SB366791 and C.C, our preliminary results showed that the treatment with these inhibitors could prevent the activation of TRPV1 channel or AMPK by bromelain in a concentration-dependent manner (Fig. S2). These results showed that TRPV1 channel and AMPK are involved in bromelain-induced NO production. However, we have not used genetic inhibition of TRPV1 channel and AMPK siRNAs to confirm this observation. Investigating the effects of heat-inactivated bromelain *in vivo* angiogenesis assay or using genetic deletion of B₂R, TRPV1, or AMPK to study the enzymatic activity of bromelain in animal models will be helpful for clarifying the protective effects of bromelain on the cardiovascular physiology and pathology.

In conclusion, our study presents a novel understanding into the molecular mechanisms responsible for the cardioprotective effects of bromelain. First, we demonstrate that the kinin system is involved in bromelain's biological action, which acts enzymatically on kininogen to release bradykinin. Second, we propose a mechanism by which bromelain activates the B₂R-Ca²⁺-TRPV1-AMPK-eNOS pathway, and the autophagy-urea cycle pathway, ultimately leading to NO biosynthesis. Finally, we reveal that releasing NO, regulated by the B₂R-Ca²⁺-TRPV1-AMPK-autophagy signaling cascade, is essential for bromelain-elicited angiogenesis. These findings provide valuable evidence to better understand bromelain's cardiovascular protective effects, expanding its potential therapeutic applications in cardiovascular diseases.

CRediT authorship contribution statement

Wen-Hua Chen: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yi-Ying Wu:** Methodology, Investigation, Formal analysis, Data curation. **Man-Chen Hsu:** Methodology, Investigation, Formal analysis, Data curation. **Chia-Hui Chen:** Methodology, Investigation, Formal analysis, Data curation. **Julia Chu-Ning Hsu:** Methodology, Investigation, Formal analysis, Data curation. **Tzong-Shyuan Lee:** Writing – review & editing, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.bioph.2025.118376](https://doi.org/10.1016/j.bioph.2025.118376).

Data Availability

Data will be made available on request.

References

- [1] J.V. Castell, G. Friedrich, C.S. Kuhn, G.E. Poppe, Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake, *Am. J. Physiol.* 273 (1 Pt 1) (1997) G139–G146.
- [2] P.A. Hu, C.H. Chen, B.C. Guo, Y.R. Kou, T.S. Lee, Bromelain confers protection against the non-alcoholic fatty liver disease in male C57bl/6 mice, *Nutrients* 12 (5) (2020).
- [3] C.H. Chen, C.C. Hsia, P.A. Hu, C.H. Yeh, C.T. Chen, C.L. Peng, C.H. Wang, T.S. Lee, Bromelain ameliorates atherosclerosis by activating the TFEB-mediated autophagy and antioxidant pathways, *Antioxidants (Basel)* 12 (1) (2022).
- [4] P.A. Hu, S.H. Wang, C.H. Chen, B.C. Guo, J.W. Huang, T.S. Lee, New mechanisms of bromelain in alleviating non-alcoholic fatty liver disease-induced deregulation of blood coagulation, *Nutrients* 14 (11) (2022).
- [5] M.A. Didamoony, A.M. Atwa, E.A. Abd, El-Haleim, L.A. Ahmed, Bromelain ameliorates D-galactosamine-induced acute liver injury: role of SIRT1/LKB1/AMPK, GSK3β/Nrf2 and NF-κB p65/TNF-α/caspase-8, -9 signalling pathways, *J. Pharm. Pharm.* 74 (12) (2022) 1765–1775.
- [6] S. Brien, G. Lewith, A. Walker, S.M. Hicks, D. Middleton, Bromelain as a treatment for osteoarthritis: a review of clinical studies, *Evid. Based Complement Altern. Med.* 1 (3) (2004) 251–257.
- [7] L.C. de Lencastre Novaes, A.F. Jozala, A.M. Lopes, V. de Carvalho Santos-Ebinuma, P.G. Mazzola, A. Pessoa, Junior, Stability, purification, and applications of bromelain: a review, *Biotechnol. Prog.* 32 (1) (2016) 5–13.
- [8] B. Juhasz, M. Thirunavukkarasu, R. Pant, L. Zhan, S.V. Penumathsa, E.R. Secor Jr., S. Srivastava, U. Raychaudhuri, V.P. Menon, H. Otani, R.S. Thrall, N. Maulik, Bromelain induces cardioprotection against ischemia-reperfusion injury through Akt/FOXO pathway in rat myocardium, *Am. J. Physiol. Heart Circ. Physiol.* 294 (3) (2008) H1365–H1370.
- [9] A.N. Fathi, M.H. Sakhaie, S. Babaei, S. Babaei, F. Slimabad, S. Babaei, Use of bromelain in cutaneous wound healing in streptozocin-induced diabetic rats: an experimental model, *J. Wound Care* 29 (9) (2020) 488–495.
- [10] R. Bahde, D. Palmes, E. Minin, U. Stratmann, R. Diller, J. Haier, H.U. Spiegel, Bromelain ameliorates hepatic microcirculation after warm ischemia, *J. Surg. Res.* 139 (1) (2007) 88–96.
- [11] S.Y. Wu, W. Hu, B. Zhang, S. Liu, J.M. Wang, A.M. Wang, Bromelain ameliorates the wound microenvironment and improves the healing of firearm wounds, *J. Surg. Res.* 176 (2) (2012) 503–509.
- [12] W.H. Chen, C.H. Chen, M.C. Hsu, R.W. Chang, C.H. Wang, T.S. Lee, Advances in the molecular mechanisms of statins in regulating endothelial nitric oxide bioavailability: interlocking biology between eNOS activity and L-arginine metabolism, *Biomed. Pharm.* 171 (2024) 116192.
- [13] P.D. Cherry, R.F. Furchtgott, J.V. Zawadzki, D. Joathanandan, Role of endothelial cells in relaxation of isolated arteries by bradykinin, *Proc. Natl. Acad. Sci. USA* 79 (6) (1982) 2106–2110.
- [14] J.R. Cockcroft, P.J. Chowienzyk, S.E. Brett, N. Bender, J.M. Ritter, Inhibition of bradykinin-induced vasodilation in human forearm vasculature by icatibant, a potent B₂-receptor antagonist, *Br. J. Clin. Pharm.* 38 (4) (1994) 317–321.
- [15] L.C. Ching, C.Y. Chen, K.H. Su, H.H. Hou, S.K. Shyue, Y.R. Kou, T.S. Lee, Implication of AMP-activated protein kinase in transient receptor potential vanilloid type 1-mediated activation of endothelial nitric oxide synthase, *Mol. Med.* 18 (1) (2012) 805–815.
- [16] K. Spengler, N. Kryeziu, S. Große, A.S. Mosig, R. Heller, VEGF triggers transient induction of autophagy in endothelial cells via AMPKα1, *Cells* 9 (3) (2020).
- [17] S.K. Park, D.T. La Salle, J. Cerbie, J.M. Cho, A. Bledsoe, A. Nelson, D.E. Morgan, R. S. Richardson, Y.T. Shiu, S. Boudina, J.D. Trinity, J.D. Symons, Elevated arterial shear rate increases indexes of endothelial cell autophagy and nitric oxide synthase activation in humans, *Am. J. Physiol. Heart Circ. Physiol.* 316 (1) (2019) H106–H112.
- [18] W.H. Chen, B.C. Guo, C.H. Chen, M.C. Hsu, C.H. Wang, T.S. Lee, Autophagy-urea cycle pathway is essential for the statin-mediated nitric oxide bioavailability in endothelial cells, *J. Food Drug Anal.* 31 (3) (2023) 519–533.
- [19] K. Katanosaka, R.K. Banik, R. Giron, T. Higashi, M. Tominaga, K. Mizumura, Contribution of TRPV1 to the bradykinin-evoked nociceptive behavior and excitation of cutaneous sensory neurons, *Neurosci. Res.* 62 (3) (2008) 168–175.
- [20] A.J. Chakraborty, S. Mitra, T.E. Tallei, A.M. Tareq, F. Nainu, D. Ciccia, K. Dhama, T. B. Emran, J. Simal-Gandara, R. Capasso, Bromelain a potential bioactive compound: a comprehensive overview from a pharmacological perspective, *Life (Basel)* 11 (4) (2021).
- [21] M.E. Errasti, A. Prospitti, C.A. Viana, M.M. Gonzalez, M.V. Ramos, A.E. Rotelli, N. O. Caffini, Effects on fibrinogen, fibrin, and blood coagulation of proteolytic extracts from fruits of Pseudananas macdonaldii, *Bromelia balansae*, and *B. hieronymi* (Bromeliaceae) in comparison with bromelain, *Blood Coagul. Fibrinolysis* 27 (4) (2016) 441–449.
- [22] S. Dave, S. Mahajan, V. Chandra, H.K. Dkhar, P. Gupta Sambhavi, Specific molten globule conformation of stem bromelain at alkaline pH, *Arch. Biochem. Biophys.* 499 (1-2) (2010) 26–31.
- [23] C. Gólias, A. Charalabopoulos, D. Stagikas, K. Charalabopoulos, A. Batistatou, The kinin system-bradykinin: biological effects and clinical implications. Multiple role of the kinin system-bradykinin, *Hippokratia* 11 (3) (2007) 124–128.

- [24] W. Feng, X. Xu, G. Zhao, J. Zhao, R. Dong, B. Ma, Y. Zhang, G. Long, D.W. Wang, L. Tu, Increased age-related cardiac dysfunction in bradykinin B2 receptor-deficient mice, *J. Gerontol. A Biol. Sci. Med. Sci.* 71 (2) (2016) 178–187.
- [25] P. Madeddu, A.F. Milia, M.B. Salis, L. Gaspa, W. Gross, A. Lippoldt, C. Emanueli, Renovascular hypertension in bradykinin B2-receptor knockout mice, *Hypertension* 32 (3) (1998) 503–509.
- [26] J.P. Schanstra, E. Neau, P. Drogosz, M.A. Arevalo Gomez, J.M. Lopez Novoa, D. Calise, C. Pecher, M. Bader, J.P. Girolami, J.L. Bascands, In vivo bradykinin B2 receptor activation reduces renal fibrosis, *J. Clin. Invest.* 110 (3) (2002) 371–379.
- [27] H.M. Siragy, A.A. Jaffa, H.S. Margolius, Bradykinin B2 receptor modulates renal prostaglandin E2 and nitric oxide, *Hypertension* 29 (3) (1997) 757–762.
- [28] S. Kumakura, I. Kamo, S. Tsurufuji, Role of bradykinin in the vascular permeability response induced by carrageenan in rats, *Br. J. Pharm.* 93 (4) (1988) 739–746.
- [29] J.B. Su, Role of Bradykinin in the regulation of endothelial nitric oxide synthase expression by cardiovascular drugs, *Curr. Pharm. Des.* 23 (40) (2017) 6215–6222.
- [30] D.J. Campbell, A. Kladis, A.M. Duncan, Bradykinin peptides in kidney, blood, and other tissues of the rat, *Hypertension* 21 (2) (1993) 155–165.
- [31] J. van Wijngaarden, R.A. Tio, W.H. van Gilst, P.A. de Graeff, C.D. de Langen, H. Wesseling, Basic pharmacology of ACE-inhibitors with respect to ischaemic heart disease: prostaglandins and bradykinin, *Eur. Heart J.* 11 (B) (1990) 84–93.
- [32] K.M. Gauthier, C.J. Cepura, W.B. Campbell, ACE inhibition enhances bradykinin relaxations through nitric oxide and B1 receptor activation in bovine coronary arteries, *Biol. Chem.* 394 (9) (2013) 1205–1212.
- [33] S. Kumakura, M. Yamashita, S. Tsurufuji, Effect of bromelain on kaolin-induced inflammation in rats, *Eur. J. Pharm.* 150 (3) (1988) 295–301.
- [34] R. Rojkjaer, A.H. Schmaier, Activation of the plasma kallikrein/kinin system on endothelial cells, *Proc. Assoc. Am. Physicians* 111 (3) (1999) 220–227.
- [35] P. Madeddu, V. Anania, P.P. Parpaglia, M.P. Demontis, M.V. Varoni, M. C. Fattaccio, N. Glorioso, Chronic kinin receptor blockade induces hypertension in deoxycorticosterone-treated rats, *Br. J. Pharm.* 108 (3) (1993) 651–657.
- [36] P. Madeddu, M.V. Varoni, D. Palomba, C. Emanueli, M.P. Demontis, N. Glorioso, P. Dassi-Fulgheri, R. Sarzani, V. Anania, Cardiovascular phenotype of a mouse strain with disruption of bradykinin B2-receptor gene, *Circulation* 96 (10) (1997) 3570–3578.
- [37] L. Cervenka, J. Maly, L. Karasová, M. Simová, S. Vítko, S. Hellerová, J. Heller, S. S. El-Dahr, Angiotensin II-induced hypertension in bradykinin B2 receptor knockout mice, *Hypertension* 37 (3) (2001) 967–973.
- [38] J.B. Calixto, Y.S. Medeiros, Effect of protein kinase C and calcium on bradykinin-mediated contractions of rabbit vessels, *Hypertension* 19 (2 Suppl) (1992) Ii87–Ii93.
- [39] A.J. Singer, S.A. McClain, B.R. Taira, J. Rooney, N. Steinhauff, L. Rosenberg, Rapid and selective enzymatic debridement of porcine comb burns with bromelain-derived Debrase®: acute-phase preservation of noninjured tissue and zone of stasis, *J. Burn Care Res.* 31 (2) (2010) 304–309.
- [40] A.N. Fathi, S. Babaei, S. Babaei, M. Baazm, H. Sakhai, S. Babaei, Effect of bromelain on mast cell numbers and degranulation in diabetic rat wound healing, *J. Wound Care* 31 (Sup8) (2022) S4–s11.
- [41] N. Akhtari, M. Ahmadi, Y. Kiani Doust Vaghe, E. Asadian, S. Behzad, H. Vatanpour, F. Ghorbani-Bidkorpeh, Natural agents as wound-healing promoters, *Inflammopharmacology* 32 (1) (2024) 101–125.
- [42] A. Weinzierl, Y. Harder, D. Schmauss, M.D. Menger, M.W. Laschke, Bromelain protects critically perfused musculocutaneous flap tissue from necrosis, *Biomedicines* 10 (6) (2022).
- [43] S. Patel, S. Srivastava, M.R. Singh, D. Singh, Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing, *Biomed. Pharm.* 112 (2019) 108615.
- [44] M.J. Malone-Povolny, S.E. Maloney, M.H. Schoenfisch, Nitric oxide therapy for diabetic wound healing, *Adv. Health Mater.* 8 (12) (2019) e1801210.
- [45] J.I. Fetterman, M. Holbrook, N. Flint, B. Feng, R. Bretón-Romero, E.A. Linder, B. D. Berk, M.A. Duess, M.G. Farb, N. Gokce, O.S. Shirihai, N.M. Hamburg, J.A. Vita, Restoration of autophagy in endothelial cells from patients with diabetes mellitus improves nitric oxide signaling, *Atherosclerosis* 247 (2016) 207–217.
- [46] N.F. Abo, El-Maggd, N.M. Ramadan, S.M. Eraky, The ameliorative effect of bromelain on STZ-induced type 1 diabetes in rats through Oxi-LDL/LPA/LPAR1 pathway, *Life Sci.* 285 (2021) 119982.
- [47] H.C. Lu, M.Y. Ng, Y.W. Liao, S. Maekawa, T. Lin, C.C. Yu, Bromelain inhibits the inflammation and senescence effect in diabetic periodontitis: a preliminary in vitro study, *J. Dent. Sci.* 18 (2) (2023) 659–665.