Betanin Prevents Experimental Abdominal Aortic Aneurysm Progression by Modulating the TLR4/NF-κB and Nrf2/HO-1 Pathways

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Betanin, a bioactive ingredient mostly isolated from beetroots, exhibits a protective effect against cardiovascular diseases. However, its effects on abdominal aortic aneurysm (AAA) have not been elucidated. In this study, an AAA model was constructed by infusion of porcine pancreatic elastase in C57BL/6 mice. Mice were then administered with betanin or saline intragastrically once daily for 14 d. Our results showed that treatment with betanin remarkably limited AAA enlargement and mitigated the infiltration of inflammatory cells in the adventitia. The increased expression of proinflammatory cytokines and matrix metalloproteinases (MMPs) was also significantly alleviated following betanin treatment. Furthermore, betanin suppressed the activation of toll-like receptor 4 (TLR4)/nuclear factor-kappaB (NF-κB) signaling in the aortic wall, and downregulated the levels of tissue-reactive oxygen species as well as circulating 8-isoprostane by stimulating the nuclear factor-E2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway. Taken together, these data suggest that betanin may attenuate AAA progression and may be used as a therapeutic drug against AAA.

Key words  betanin; abdominal aortic aneurysm; toll-like receptor 4 (TLR4); reactive oxygen species; nuclear factor-E2-related factor 2 (Nrf2)

INTRODUCTION

Abdominal aortic aneurysm (AAA), a life-threatening disease, is defined as the development of irreversible localized aortic dilatation that exceeds 50% of the normal diameter.1) Although most patients with AAA present with no clinical manifestations at the early stage, the disease is frequently fatal following rupture of the aneurysm.2,3) Clinically, endovascular techniques and open surgery are regarded as standard therapies for the prevention of aortic rupture, but these render patients susceptible to a high risk of perioperative morbidity and mortality.4,0) Thus, there is an urgent need to identify suitable non-invasive pharmaceuticals to intervene with AAA progression.

It is well known that the main pathological characteristic of AAA is sterile inflammation.7) Toll-like receptors (TLRs) are a family of surface molecules that are activated in innate immunity and chronic inflammation.9) Notably, TLR4 can be activated by exogenous and endogenous triggers to culminate in the generation of various proinflammatory cytokines by initiating downstream signaling molecules, such as nuclear factor-kappa B (NF-κB).9) Numerous reports have shown that TLR4 expression is upregulated in patients with AAA.10,11) Therefore, blockade of the TLR4/NF-κB signaling pathway has emerged as an effective strategy for treating AAA.

Reactive oxygen species (ROS), are generated from cellular metabolism as normal by-products.12) Physiological levels of ROS are involved in many cellular processes, including repair, autophagy, survival, and differentiation.13) Further, oxidative stress resulting from excessive ROS production or deficiency of antioxidant system, has been implicated in AAA malignant progression by triggering oxidative damage and mediating intracellular signaling cascades.14) Accumulating evidence indicates that excessive ROS caused irreversible cellular damage and facilitates the release of plentiful proinflammatory cytokines and matrix metalloproteinases (MMPs), thus contributing to matrix degradation and depletion of vascular smooth muscle cells (VSMCs), ultimately resulting in aortic remodeling.15) Hence, it is reasonable to anticipate that antioxidative drugs may have the potential to inhibit AAA development by eliminating ROS.

In the past few decades, the application of natural compounds has been observed to protect against various cardiovascular diseases.16) Betalains are nitrogen-containing pigments, and can be extracted from beetroots17,18) (Fig. 1A). Betanin (betanidin-5-O-glucoside, Fig. 1B), one of the most abundant compounds among betalains, acts as a natural red colorant in food products, medicinal products, and cosmetics.19) Previous studies have reported that betanin can reduce inflammation, oxidative stress, and apoptosis in several diseases, such as myocardial damage, atherosclerosis, and acute kidney damage.20–24) However, whether it helps to prevent AAA progression remains unknown. Herein, we investigated the roles of betanin in a mouse model of AAA and elucidated its underlying mechanisms to provide the theoretical foundation for further research.

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MATERIALS AND METHODS

Mice Healthy 8-week-old male C57BL/6 mice were procured from Charles River Company (Beijing, China) and raised under standard conditions (22 ± 3 °C; 55 ± 10% relative humidity) with a 12-h light–dark cycle in the Animal Experimental Center of Shandong Provincial Hospital. The animal experiments were approved by Ethical Review Board of Shandong University and complied with guidelines outlined in the Guide for the Care and Use of Laboratory Animals.25

Porcine Pancreatic Elastase-Induced Murine AAA
Thirty mice were randomly divided into three groups (n = 10 per group), namely normal, AAA and betanin. The AAA model was induced by infusing porcine pancreatic elastase (PPE; Sigma, St. Louis, MO, U.S.A.) into the abdominal aorta, as per methods described previously.26 Briefly, mice were anesthetized using isoflurane inhalation and placed on an operating table. A laparotomy was performed to separate the infrarenal abdominal aorta from the inferior vena cava. All lumbar arteries were ligated using 9-0 lines. After the proximal and distal aorta was ligated using 6-0 silk suture temporarily, a heat-tapered segment of a polyethylene catheter (PE-10) was inserted into the controlled abdominal aorta. Subsequently, the mice of AAA group and betanin group were perfused with 1.5U/mL PPE, and the mice of normal group were perfused with the same dose of saline through a catheter. This experimental setup was maintained for 5 min at a constant pressure of 100 mmHg. Next, the hot conical PE-10 polyethylene tube was removed, and the aorta’s puncture port was sutured with a 10–0 thread. The distal and proximal ligatures of the artery were subsequently removed and the abdominal wall was sutured in a layer-by-layer manner. Mice were then placed in the cage and given standard experimental food and water ad libitum until they fully recovered from the effects of anesthesia.

Betanin Treatment
Briefly, betanin was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan, CAS:7659-95-2, Catalogue No. B0397) and dissolved in saline (ab97779, Abcam). Standard stock solution of betanin (10 mg/mL) was prepared in 1.5% ethanol (ab125212, Abcam) and stored at −20 °C. After surgery, mice in the betanin group were intragastrically administered with betanin (100 mg/kg). Mice in the normal group and AAA groups were intragastrically administered with the same dose of saline once daily for 14 d. The dosage of betanin was ascertained based on a former study wherein betanin was shown to effectively alleviate oxidative stress and inflammatory responses in the kidneys of paraquat-treated rats.27

Ultrasonic Measurement of Mouse Aortic Size
Before PPE infusion (day 0) and on days 3, 7, and 14 after PPE infusion, mice were fixed on an animal platform, and scanned transversely to record the maximal diameter of aneurysm. Representative images were obtained using the B-mode ultrasound system (Vevo 2100, Visual Sonics Inc., Toronto, Canada) with a central frequency of 40 MHz.

Enzyme-Linked Immunosorbent Assay (ELISA)
To assess systemic oxidative stress, the content of 8-isoprostane in serum, a proposed biomarker of antioxidant deficiency and oxidative stress,28 was analyzed using a commercially available ELISA kit (ab175819; Abcam, Cambridge, MA, U.S.A.) following manufacturer’s instructions. The absorbance of samples was measured via a microplate reader (MultiskanGO, Thermo, U.S.A.) at 450 nm.

Histopathology
The 4% paraformaldehyde-fixed abdominal aortic tissue was imbedded in the Tissue-Tek CRYO-O.C.T. Compound (4583, Sakura Finetek USA, U.S.A.) and prepared as 4-µm-thick frozen sections. Hematoxylin–eosin (H&E) staining was applied to assess morphological changes, and Elastica van Gieson (EVG) staining was used for elastic lamina detection. Images were observed by using a confocal laser scanning microscope (Nikon, Tokyo, Japan).

Elastin degradation scores were evaluated as per previously described methods in the following manner: score 1, none or mild; score 2, moderate; score 3, moderate to severe; and score 4, severe elastin degradation.29

Immunofluorescence
ROS expression of in the abdominal aortic wall from different groups was compared using immunofluorescence (IF) staining. Cryostat sections were incubated in ROS reagent (G1045, Servicebio, Wuhan, China) at 4 °C for 30 min and in 4’-6-diamidino-2-phenylindole (DAPI) (G1012, Servicebio) at room temperature for 10 min. The slices were scanned using confocal laser scanning microscope.

Immunohistochemistry
Anti-α-smooth muscle actin (α-SMA; ab5694, Abcam) was used to detect VSMCs. Antibodies against CD31, CD68, CD8, and B220 (ab28364, ab22378, ab64110, ab125212; Abcam) were used to analyze neovascularization and inflammatory cell infiltration of the abdominal aortic wall. The local expression of MMPs and oxidative stress were analyzed by antibodies against MMP-2 (ab97779, Abcam), MMP-9 (ab228402, Abcam), and nuclear factor erythroid 2-related factor 2 (Nrf2; 216396-1-AP, Proteintech, Wuhan, China).

For the immunohistochemical (IHC) staining of the mouse aortic tissue, cross-sections were rinsed thrice in phosphate buffered saline (PBS) and subsequently incubated with pri-
Primary antibodies for 1 h at 37°C. Sections were then labeled with secondary antibodies for 30 min at room temperature, followed by 30 min incubation with biotin. Immunocomplexes were observed using the AEC Peroxidase Substrate Kit (A2010, Solarbio, Beijing, China) and imaged on a confocal laser scanning microscope (Nikon). Immunostaining images were analyzed using the Image-Pro Plus software.

Quantitative Real-Time RT-PCR
The expression of interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α in abdominal aorta was examined using quantitative real-time RT-PCR (qRT-PCR). Total RNA was isolated from abdominal aorta tissue with Trizol Reagent (NR0002, Leagene, Beijing, China), reversely transcribed into cDNA using the Strand cDNA Synthesis Kit (6130, TaKaRa, Japan), and amplified with the Power SYBR Green PCR Mastermix (RR420A, TaKaRa) following the manufacturer's instructions. Associated genes were quantified by qRT-PCR using the Roche 480 real-time PCR system (Roche Diagnostics, Switzerland). The primers sequences are listed in Table 1.

Western Blot
Protein expression of MMP-2, MMP-9, TLR4, NF-kB-p-P65, Nrf2, and heme oxygenase-1 (HO-1) was detected as follows: The total proteins were extracted from abdominal aorta tissue homogenates. The bicinchoninic acid protein assay kit (ab102536, Abcam) was applied to determine the protein concentration. Subsequently, protein samples were separated with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 60 min, and then transferred onto polyvinylidene difluoride membranes; the transfer procedure lasted for 90 min. The membranes were incubated with antibodies against MMP-2 (ab97779, Abcam), MMP-9 (ab228402, Abcam), TLR4 (ab22048, Abcam), NF-kB-p-P65 (3036, CST, U.S.A.), Nrf2 (16396-1-AP, Proteintech), HO-1 (27282-1-AP, Proteintech), and β-actin (ab8226, Abcam) at 4°C overnight. The next day, the membranes were rinsed, followed by incubation with secondary antibodies at room temperature for 1 h. Proteins were detected using enhanced...
chemiluminescence reagents (Amersham Biosciences, U.S.A.), and quantitatively analyzed using the ImageJ software.

**Statistical Analysis** All data are expressed as the mean ± standard deviation (S.D.). Statistical significance between groups was analyzed using the unpaired Student’s *t*-test or two-way ANOVA. *p* < 0.05 was considered statistically significant.

**RESULTS**

**Betanin Limits the Enlargement of Experimental AAA** Representative images of maximal aortic diameter on days 0, 3, 7, and 14 after PPE infusion are presented in Fig. 2A. We found that the maximal aortic diameter in the AAA group was remarkably augmented compared with the normal group (AAA group, 1.467 ± 0.102 mm vs. normal group, 0.601 ± 0.018 mm), but betanin treatment reduced the maximal aortic diameter (betanin group, 1.100 ± 0.094 mm) on day 14 post-PPE surgery (Fig. 2B). Particularly, the growth of aortic diameters in betanin group began to decelerate from the 3rd day, compared with the AAA group (Fig. 2C). Ultimately, the incidence of AAA in the normal and AAA groups was 0% (0/10) and 100% (10/10), respectively. After the betanin administration, the incidence of AAA in betanin group was decreased to 50% (5/10) (Fig. 2D). Based on these data, we demonstrated that betanin could effectively limit aortic expansion, thus preventing the development of aneurysms.

**Betanin Repairs the Histopathological Lesion of the Aneurysm Wall** The main pathologic changes in AAA include adventitia thickening, elastin degradation, VSMC loss, and neovascularization. We next performed H&E, EVG, and immunohistochemistry (IHC) assays to assess the histological changes in the AAA following betanin treatment. As shown in Fig. 3A, the AAA group exhibited a severe damage to media and thickened adventitia compared with that in the normal group. However, these pathological features were alleviated after the administration of betanin. EVG staining revealed that betanin attenuated the disruption and degradation of elastic laminae (Fig. 3B). In addition, betanin upregulated the level of α-SMA and reduced the expression of CD31 to a certain degree, indicating that it could reverse the destruction of VSMCs and inhibit neovascularization in the aortic wall (Figs. 3C and 3D).

![Fig. 3. Effect of Betanin on the Histopathological Lesion of the Aortic Wall](image-url)
Overall, these results suggest that betanin effectively restrained AAA progression.

**Betanin Inhibited Inflammatory Responses in the Adventitia** It is well known that inflammatory cells, including macrophages and lymphocytes, have a contributory effect on AAA formation by secreting proinflammatory cytokines. Accordingly, the expression of CD68+ macrophages, CD8+ T cells, and B220+ B cells were detected using IHC. IHC results further revealed that the positive expression of CD68+ macrophages, CD8+ T cells and B220+ B cells in the AAA group was distinctly augmented compared with that in the normal group, while these changes were inhibited after betanin administration (Fig. 4A). Further, the elevated levels of IL-1β, IL-6, and TNF-α were sufficiently suppressed after betanin treatment (Fig. 4B). In a word, betanin could effectively attenuate inflammatory responses in the aneurysm wall.

**Betanin Suppresses MMPs and the TLR4/NF-κB Pathway** Increased expression of MMPs, particularly MMP-2 and MMP-9, leads to gradual damage of the extracellular matrix (ECM), further resulting in aortic remodeling. We first conducted an IHC assay to measure the levels of MMP-2 and MMP-9. The findings revealed that the elevated positive expression of MMP-2 and MMP-9 in the aortic aneurysms was obviously down-regulated after the betanin administration (Figs. 5A, B). Similar results were observed in the Western blot assay (Figs. 5C, D). Growing evidence has shown that the activation of the TLR4/NF-κB signaling may enhance MMP activity and result in aneurysm formation. Thus, we suspected that betanin might prevent AAA formation through regulation of the TLR4/NF-κB pathway. As shown in the Figs. 5E, F, the protein levels of TLR4 and p-P65 in the aortic aneurysms were notably increased compared with that in the normal group, whereas their protein expression were reversed after betanin treatment. Intriguingly, betanin had no effect on the mRNA level of TLR4, which is due to regulation of betanin on the TLR4 translation. In a word, the suppression of TLR4/NF-κB signaling was a potential mechanism underlying the anti-inflammatory effect of betanin.

**Betanin Attenuates Oxidative Stress and Activates the Nrf2/HO-1 Pathway** Previous reports have demonstrated that oxidative stress is implicated in AAA pathogenesis. Therefore, we examined the levels of tissue-ROS and circulating 8-isoprostane. The IF assay showed that betanin treatment markedly decreased the ROS generation in the aneurysm wall (Figs. 6A, B). Furthermore, the levels of circulating 8-isoprostane in the betanin group were markedly lower than that in the AAA group, as shown by ELISA (Fig. 6C). It is well known that increased ROS levels depend not only on the

Fig. 4. Effect of Betanin on Inflammatory Responses in the Aortic Wall
(A) Representative images of immunostaining and densitometric analysis of CD68, CD8, and B220 in the infrarenal aortic wall. (B) Expression of IL-1β, IL-6, and TNF-α in the infrarenal aortic wall. *p<0.05, **p<0.01, ****p<0.0001 vs. the normal group; †p<0.05, ‡p<0.01, §§§p<0.0001 vs. the AAA group.
excessive generation of ROS, but also on the deficiency of antioxidative systems. Thus, we explored the effects of betanin on Nrf2/HO-1 expression in aortic tissues. Data revealed that betanin treatment markedly increased the levels of Nrf2 and HO-1 proteins, but the mRNA level of Nrf2 was not significantly changed by betanin (Figs. 6D–F), which indicating that betanin could protect the aortic wall from ROS-induced injury by activating the Nrf2/HO-1 pathway.

DISCUSSION

AAA is a progressive abdominal aortic dilation due to abnormal interactions between genetic factors and the environment.38) Emerging evidence suggests that male, age, smoking, and hypertension are common risk factors for AAA development.39,40) Despite the considerable advances in surgical treatment, the prognosis of ruptured AAs remains poor, with high mortality rates.3) Unfortunately, there are no effective strategies that can limit aneurysm enlargement in the early stage of AAA. Therefore, development of new drugs is required for AAA treatment.

Betanin has garnered increasing attention owing to its safety, affordable cost, accessibility, and biodegradability.41) Previous studies have indicated that betanin and betanin-rich diets exert anti-inflammatory and anti-oxidative effects in cancer, neurodegenerative diseases, ischemic damage, and inflammatory disorders.42–45) Considering that the main mechanisms involved in AAA pathogenesis are associated with inflammatory responses and oxidative stress, we investigated the role of betanin in experimental AAA using a PPE-induced C57BL/6 mouse model, which closely resembled human infrarenal AAA.38,46,47)

Increased infiltration of macrophages and lymphocytes into the aneurysm wall has been reported to be the predominant part of the inflammatory response, which is a distinguishing feature in AAA pathogenesis.46,48,49) Inflammatory cells secrete MMPs and various proinflammatory chemokines, such as IL-1β, IL-6, and TNF-α, which in turn recruit more inflammatory cells to the injured site, further forming an inflammatory circuit.50,51) Additionally, increased MMP expression is found in AAA, which facilitates aortic remodeling via degradation of the ECM. In this study, we confirmed that betanin could significantly alleviate the inflammatory cell infiltration and downregulate the levels of MMP-2 and MMP-9, which indicated that betanin treatment restrained the inflammatory response and protected elastic laminae and VSMCs from destruction in PPE-induced AAA.

As a sensory receptor of various inflammatory stimuli, TLR4 initiate downstream signaling cascades, such as myeloid differentiation factor 88 (MyD88) and NF-κB, thus mediating inflammatory responses in animal models. NF-κB, a crucial transcription factor, is involved in the induction of

Fig. 5. Effect of Betanin on MMPs and the TLR4/NF-κB Pathway in the Aortic Wall

(A, B) Representative images of immunostaining and densitometric analysis of MMP-9 and MMP-2 in the infrarenal aortic wall. (C–E) Representative blots and semiquantitative analysis of MMP-9, MMP-2, TLR4, and NF-κB-p-P65 in the infrarenal aortic wall. (F) Expression of TLR4 in the infrarenal aortic wall.*p < 0.05, ***p < 0.001, ****p < 0.0001 vs. the normal group; #p < 0.01, ###p < 0.001, ####p < 0.0001 vs. the AAA group.
inflammatory injury.\textsuperscript{52} Activated p65 is phosphorylated and then translocated into the nucleus to regulate the expression of proinflammatory cytokines.\textsuperscript{53} Increasing evidence shows that the TLR4/NF-κB axis is a vital signaling component associated with the occurrence and development of AAA.\textsuperscript{54} Huggins et al. revealed that a TLR4 antagonist could lessen the damage severity in AAA.\textsuperscript{55} Therefore, targeting the TLR4/NF-κB axis may represent a potential intervention for AAA therapy. In this research, we evaluated the effects of betanin on the TLR4/NF-κB pathway. The results revealed that TLR4 expression and the phosphorylation level of p65 were downregulated following betanin administration, which provided novel insights into the anti-inflammatory activity of betanin.

ROS comprise highly reactive oxygen-containing molecules, such as the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (·OH).\textsuperscript{56} They are normal metabolic products within the body, and intracellular ROS levels closely rely on multifarious synthesis processes and antioxidant defenses.\textsuperscript{57} A moderate level of ROS is involved in maintaining cellular function and homeostasis. However, once the redox status changes to oxidation, excessive ROS generation will induce oxidative stress, followed by infliction of oxidative injury in DNA, lipids, and proteins, eventually contributing to the progression of various diseases.\textsuperscript{58} Former studies have revealed that ROS overexpression is implicated in ECM remolding as well as VSMC apoptosis, which is regarded as a prerequisite for AAA.\textsuperscript{59} Nrf2, a vital antioxidant initiator, is transferred into the nucleus and combined with the antioxidant response elements in the promoter regions of antioxidative downstream genes, including HO-1, superoxide dismutase (SOD) and catalase, thus alleviating the deleterious effects of oxidative stress.\textsuperscript{60} In this study, we confirmed that betanin augmented the levels of Nrf2 and HO-1, followed by scavenging of excessive ROS and reduction of 8-isoprostane. Although the mRNA levels of TLR4 and Nrf2 did not change significantly after betanin treatment, we thought that betanin could mitigate the expression of TLR4 and Nrf2 at post-transcriptional levels. However, the relationship between the

![Fig. 6. Effect of Betanin on Oxidative Stress in the Aortic Wall](image-url)

(A) Representative images of ROS in the infrarenal aortic wall. (B) Semiquantitative analysis of ROS in the infrarenal aortic wall. (C) Relative expression of 8-isoprostane in the sera of mice. (D) Expression of Nrf2 in the infrarenal aortic wall. (E) Representative blots and semiquantitative analysis of Nrf2 and HO-1 in the infrarenal aortic wall. (F) Densitometric analysis and representative images of immunostaining of Nrf2 in the infrarenal aortic wall. *$p<0.05$, **$p<0.01$, ****$p<0.0001$ vs. the normal group; "#$p<0.01$, ####$p<0.0001$ vs. the AAA group.
Nrf2/HO-1 pathway and TLR4/NF-κB signaling regulated by betanin deserves further exploration.

CONCLUSION

Our results demonstrated that betanin exerted anti-inflammatory activity and ROS scavenging ability, thereby retarding AAA progression. Mechanistically, the inhibitory effect of betanin on AAA formation is possibly associated with the suppression of TLR4/NF-κB signaling and the activation of the antioxidative Nrf-2/HO-1 pathway. Collectively, these findings provide reliable evidence that betanin may be a promising candidate drug against human AAA, and future research should be conducted to explore its mechanisms of action.

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Conflict of Interest The authors declare no conflict of interest.

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