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Article

Vasoconstrictive effects of betanin on isolated preparations of rat aorta

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Betanin is a natural pigment belonging to the group of betalains with vasoconstrictive properties. This study investigated the underlying mechanism involved in the vasoconstrictive effect of betanin in rat aorta. Betanin enhanced the contractile response in aortic rings precontracted with phenylephrine. In aortic preparations under resting tonus, betanin induced potent and sustained contractions. The contractile effect of betanin was increased in endothelium-denuded aortic rings, while treatment of endothelium-intact preparations with the alpha-1-adrenergic receptor antagonist prazosin abolished the contractile effects of betanin. In the presence of verapamil, the contractile effects of betanin were decreased, and they were abolished under Ca^{2+}-free solution. Vasoconstriction was not affected by treatment with yohimbine and guanethidine. Betanin partially restored the endothelium-dependent vasorelaxant effect induced by acetylcholine, a response that was blunted in aortic rings treated with baicalin, an inhibitor of the nitric oxide (NO) - cyclic guanosine monophosphate (cGMP) - protein kinase G (PKG) pathway. In conclusion, the vasoconstrictive effects of betanin likely involve alpha-1 adrenoceptors and are dependent on endothelial modulation.

Keywords: Betanin. Contractile effect. Endothelium function. Vascular smooth muscle.

INTRODUCTION

Betanin is a water-soluble nitrogenated heterocyclic compound found in plants, with its most important natural source being the root of red beets (*Beta vulgaris* L.). It belongs to a class of natural pigments called betalains, which are synthesized in plants from the amino acid tyrosine to yield betalamic acid. This acid can be converted to betacyanins, including betanin (Delgado-Vargas, Jimenez, Paredes-Lopez, 2000; Silva *et al.,* 2022). Betanin has gained attention as a bioactive compound with the capacity to inhibit lipid membrane and low-density lipoprotein (LDL) peroxidation, modulate reactive oxygen species (ROS) generation, influence gene expression to reduce the release of inflammatory cytokines, and enhance antioxidant enzyme activities (Hobbs *et al*., 2012).

Beetroot, a betanin-rich vegetable, can lower blood pressure in adults, likely due to its vasodilatory properties (Hobbs *et al*., 2012; Jonvik *et al*., 2016). In fact, Esatbeyoglu *et al.* (2015) demonstrated, in vitro, a concentration-dependent vasorelaxation induced by concentrated red beet juice on endothelium-intact pocine arterial rings. This vasodilatory effect is thought to be partially driven by an increase in cyclic guanosine monophosphate (cGMP), a process dependent on nitric oxide (NO), which, in turn, relaxes smooth muscle cells. This is a plausible hypothesis considering that red beetroot is a major dietary source of nitrate, a physiological substrate for NO production (Baião *et al*., 2017).

In contrast to the findings with concentrated red beet juice, experimental approaches using betanin in isolated preparations of vascular smooth muscle did not induce vasorelaxation. In fact, Tawa *et al.* (2020) showed that betanin induced vasoconstriction in mesenteric and pulmonary arteries of pigs. The precise mechanisms

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underlying this vasoconstrictor effect, however, remain unclear. The present study aims to evaluate how betanin induces its vasoconstrictive effects on isolated preparations of rat aorta.

MATERIAL AND METHODS

Animals

Wistar rats weighing between 200 and 280 g were obtained from the vivarium maintained by the Department of Physiology and Pharmacology at the Federal University of Ceará in Fortaleza, Brazil. The animals were previously housed in polypropylene cages measuring 410 × 340 × 160 mm, with six rats per cage, and subjected to a 12:12-hour light-dark cycle. They had ad libitum access to both food and water. All procedures in this study were previously submitted to and approved by the local animal ethics committee (CEUA No. 23091.010411/2019-08).

Contractile studies in isolated rat aorta

To prepare the isolated aortic segments, the animals underwent anesthesia using 2,2,2-tribromoethanol (250 mg/kg) and were sacrificed through exsanguination. Following laparotomy, the descending thoracic aorta was carefully extracted and then cross-sectionally sliced into cylindrical ring-shaped segments measuring 1 mm x 5 mm. These segments were subsequently affixed to triangular supports made of steel wire and suspended in organ baths, each containing 5 mL of Modified Krebs-Henseleit solution (MKHS). The rings were gently stretched to a passive tension of 1 g, and the tension applied was continuously monitored by means of an isometric force transducer (ML870B60/C-V, AD Instruments, Australia) connected to a data acquisition system (PowerLab 8/30, AD Instruments).

Solutions, drugs and experimental protocols

The MKHS solution was composed of the following mM concentrations: NaCl 118.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 11.1. It was maintained at a pH of 7.4 and continuously aerated at 37 °C with a mixture of 95% O_2 and 5% CO_2. All chemicals, including betanin, acetylcholine (ACh), phenylephrine (Phe), baicalin, prazosin, guanethidine, yohimbine, and verapamil, were sourced from Sigma-Aldrich (St. Louis, MO, USA). These reagents were initially prepared as concentrated stock solutions in physiological saline containing 5% dimethyl sulfoxide (DMSO) and then further diluted to reach the desired concentration in the bath chamber. Before use, the solutions were sonicated. The final concentration of the vehicle in the organ bath did not exceed 0.02% (v/v).

For experiments requiring Ca^{2+}-free conditions, $CaCl_2$ was omitted from the standard MKHS solution, and ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) was added at a concentration of 1 mM. In some aortic tissues, the endothelium was gently removed immediately after dissection by rubbing the aortic lumen with a stainless-steel wire. Preparations with and without endothelium were pre-contracted with Phe (0.1 μM) at the start of the experiment. After establishing a contractile plateau, they were challenged with 1 μM ACh. The absence of a vasorelaxant response to ACh served as evidence that the endothelium had been effectively removed. Following an equilibration period of at least 60 minutes, control contractions were triggered by adding a submaximal concentration (60 mM) of KCl to the bath. Preparations were considered equilibrated when two successive control contractions displayed similar amplitudes.

Statistical analysis

Data are presented as mean ± the standard error of the mean (SEM), and n represents the number of experiments. Values of the median effective concentration (EC_{50}) were calculated by semi-logarithmic interpolation and expressed as geometric mean and 95% confidence interval. One- or two-way analysis of variance (ANOVA) followed by the Holm-Sidak test were used to compare the groups. Statistical significance was considered when $p < 0.05$.

RESULTS

Contractile effects of betanin on isolated rat aorta

In the first set of experiments, aortic rings with intact endothelium produced a sustained contractile response to the addition of a single concentration of phenylephrine (1 μM; 1.3 ± 0.1 g; n = 5). A cumulative addition of betanin (1 – 3000 μM; Figure 1A) caused a slight but significant increase in the magnitude of the phenylephrine-induced contraction to a value of 122.8% ± 6.0% ($p < 0.05$, Holm-Sidak test, n = 5, Figure 1B) of the response recorded with phenylephrine alone.

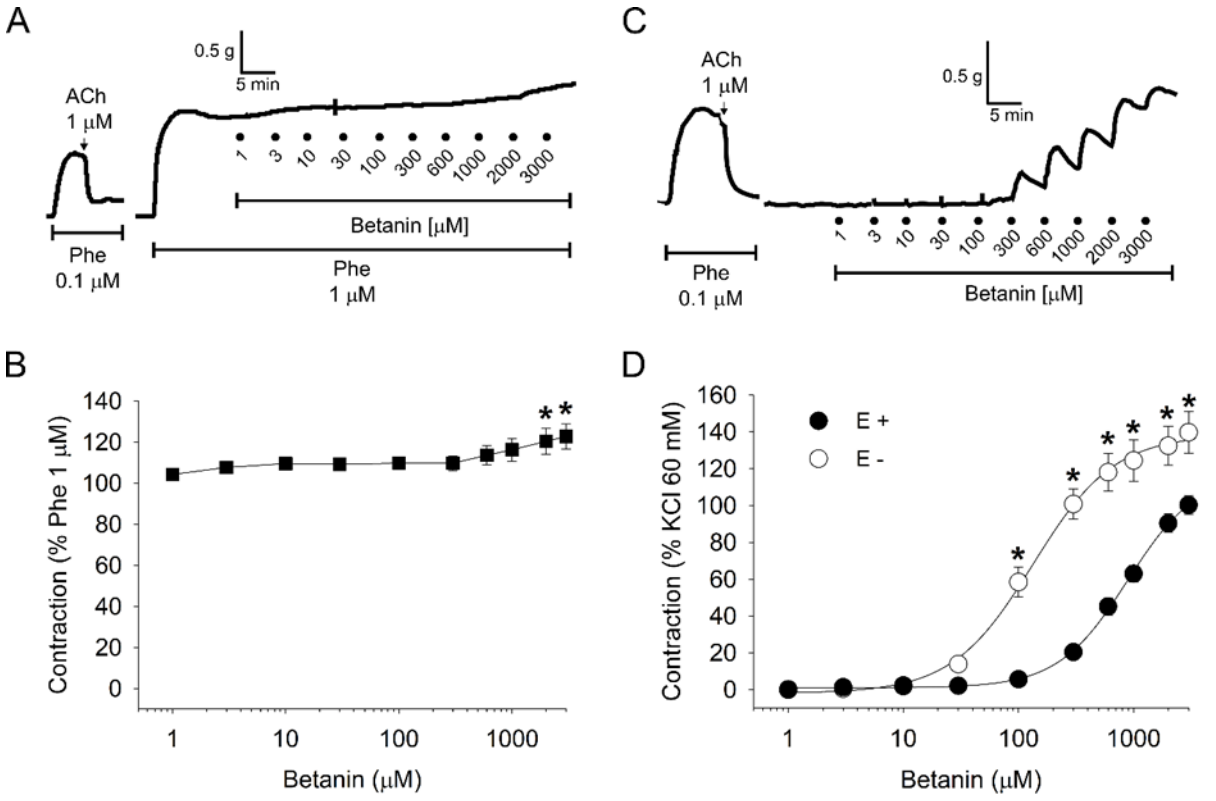

FIGURE 1 - Betanin-induced contractile effect on rat aorta.

Panels A and C show representative experiments of the effects caused by cumulative addition of betanin (1–3000 μM) on aortic rings. Acetylcholine (ACh; 1 μM) relaxed an initial contraction induced by phenylephrine (Phe; 0.1 μM), confirming the presence of intact endothelium. Aortic rings were then washed and betanin was added: in Panel A, during a sustained Phe (1 μM) contraction; in Panel C, under resting tone. Panel B depicts a graph demonstrating that cumulative betanin (1–3000 μM; n = 5) caused a slight but significant increase in the contraction of pre-contracted aortic rings with Phe. Under resting conditions (Panel D), betanin (1–3000 μM; n = 8) concentration-dependently contracted both endothelium-intact (E+) and denuded (E-) aortic rings. Data are expressed as a percentage of the initial contraction induced by Phe (1 μM) in Panel B or KCl (60 mM) in Panel D. Data represent mean ± standard error of the mean (SEM). *$p < 0.05$, Holm-Sidak test, compared to values before betanin addition (Panel B) or E+ contractions (Panel D).

In another set of experiments with isolated preparations of endothelium-intact rat aorta maintained under resting tension (Figure 1C), the cumulative addition of betanin (1 – 3000 μM) induced a concentration-dependent contraction response ($p < 0.05$, Holm-Sidak test, n = 8) with an EC_{50} of 687.3 [499.8 – 944.9] μM and an Emax of 100.3% ± 5.0% of a reference contraction induced by 60 mM KCl (data not shown). The maximal effect of betanin was observed at 3000 μM (E+, representing preparations with intact endothelium; Figure 1D).

In tissues subjected to mechanical denudation of the endothelial layer (E-, representing preparations without endothelium; Figure 1D), the contractile effect induced by the same concentration range of betanin was significantly enhanced, as observed by a significant decrease in the EC_{50} value to 85.7 [55.0 – 133.7] μM ($p<0.05$, Mann-Whitney test; n = 5) and a maximal effect of 139.8% ± 11.3% in relation to the control ($p<0.05$).

The contractile effect of betanin was abolished by the alpha-1 adrenergic receptor antagonist prazosin

In aortic rings with intact endothelium, treatment with guanethidine (1 μM), an inhibitor of catecholamine release, or with yohimbine (30 nM), an antagonist of alpha-2 receptors, did not significantly alter the contractile response induced by betanin (1 - 3000 μM) ($p > 0.05$, two-way ANOVA, Figure 2). In contrast, pretreatment of aortic rings with the alpha-1-adrenergic receptor blocker prazosin (10 μM) completely inhibited the contractile effect elicited by betanin ($p<0.05$, Holm-Sidak test, Figure 2).

The contractile effects of betanin on aortic rings depended on extracellular Ca^{2+} levels and endothelial function

In one set of experiments with endothelium-intact aortic rings maintained initially in Ca^{2+}-free medium, the cumulative addition of betanin (1– 3000 μM) did not induce significant contractile effects on aortic rings ($p < 0.05$, Holm-Sidak test, n = 7, Figure 3B). In contrast, pretreatment of endothelium-intact aortic rings with the voltage-gated calcium channel blocker verapamil (1 μM) in 2.5 mM Ca^{2+}-containing MKHS significantly reduced the maximal magnitude of the betanin-elicited contraction to 72.75% ± 6.7% of the reference contraction induced by 60 mM KCl ($p < 0.05$, n = 5, Figure 3A).

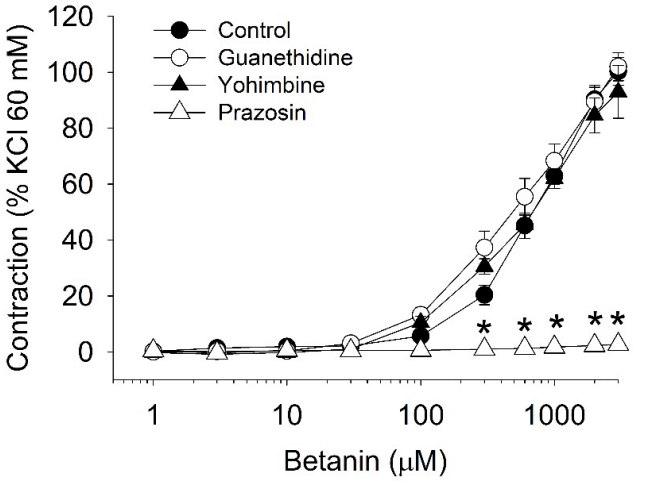

FIGURE 2 - Characterization of betanin's contractile effects on rat aorta with adrenergic receptor antagonists.

Concentration-response curves were generated for betanin (1–3000 μM) in endothelium-intact aortic rings under various conditions: control (no antagonist, n = 8), guanethidine (1 μM, n = 5), yohimbine (30 nM, n = 5), or prazosin (10 μM, n = 5). Data are expressed as a percentage of the reference contraction induced by KCl (60 mM) and represent mean ± standard error of the mean (SEM). *$p < 0.05$, Holm-Sidak test, compared to the control curve (betanin alone).

Betanin partially restored the impaired endothelium-dependent vasorelaxant response to acetylcholine in aortic rings treated with baicalin

In aortic rings with intact endothelium that were previously contracted with Phe (0.3 μM), the addition of ACh (0.01 – 30 μM) relaxed the Phe-induced contraction to just 12.1% ± 2.8% of its control value recorded before the addition of ACh (at 30 μM). In the presence of baicalin (100 μM), an inhibitor of the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) pathway, the vasorelaxant response to ACh was significantly reduced, and the Phe-elicited contraction relaxed only to 51.7% ± 7.2% of its control value ($p<0.05$, Holm-Sidak test, Figure 4D).

In aortic rings pretreated with betanin (1000 μM), the deleterious effect of baicalin (100 μM) on the cholinergic response was partially blunted, and the aortic preparations relaxed to 30.9% ± 4.7% of the control contraction after the addition of ACh (0.01 – 30 μM), a response that was significantly higher compared to that observed in the absence of betanin ($p < 0.05$, Holm-Sidak test, Figure 4D).

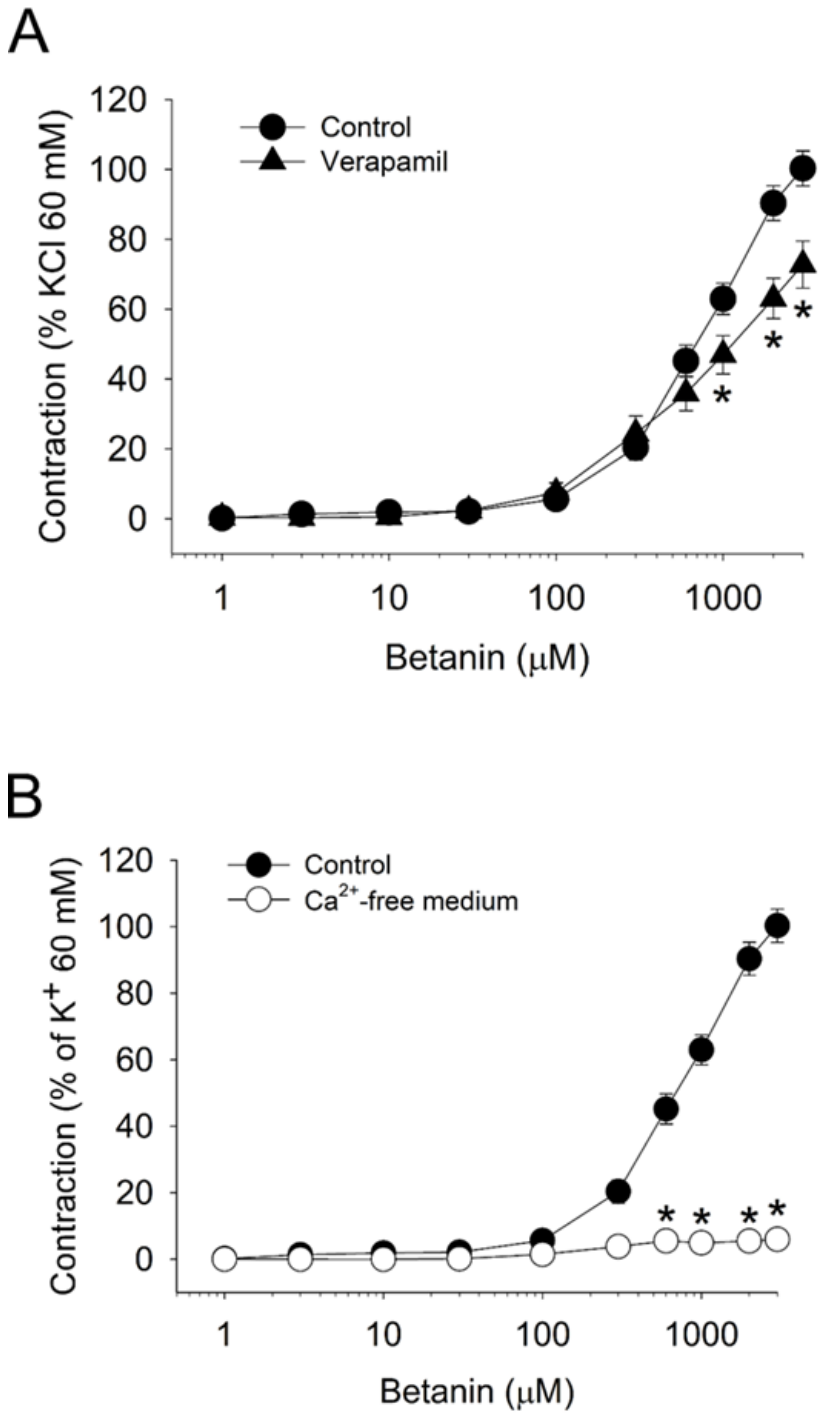

FIGURE 3 - Verapamil and calcium depletion inhibit betanin's contractile effect on isolated rat aorta.

Panel A: Concentration-response curves for betanin (1–3000 μM) were generated in endothelium-intact aortic rings maintained in calcium-containing medium: control (no verapamil, n = 8) and with verapamil (1 μM, n = 5). Panel B: Concentration-response curves for betanin (1–3000 μM) were generated in endothelium-intact aortic rings, either with calcium-containing medium (control) or calcium-free medium. Removing extracellular calcium abolished betanin's contractile effect. Data expressed as a percentage of the reference contraction induced by KCl (60 mM) and represent mean ± standard error of the mean (SEM). #, *, $p < 0.05$, Holm-Sidak test, compared to the control curve

DISCUSSION

This investigation examined the vasoconstrictive effects triggered by betanin, a pigment of the betalain class found in red beetroot, a vegetable that has recently gained attention for its potential to lower blood pressure (Sagar *et al.*, 2023; Wei *et al.*, 2023). The hypotensive effects of beetroot appear to be related to the ingestion of its high levels of dietary nitrate (NO_3^-) and its subsequent conversion to nitrite (NO_2^-), which generates the vasodilator nitric oxide (NO) (Lundberg, Weitzberg, Gladwin, 2008). However, Tawa *et al.* (2020) demonstrated that betanin, a constituent of beetroot, did not induce vasorelaxant effects in mesenteric arteries of pigs. This finding argues against a potential role of betanin in the beetroot-induced blood pressure-lowering effect. Consistent with these observations, the present study in rat aorta confirmed that betanin did not relax a sustained contraction induced by phenylephrine, an alpha-adrenergic receptor agonist (Zhong, Minneman, 1999).

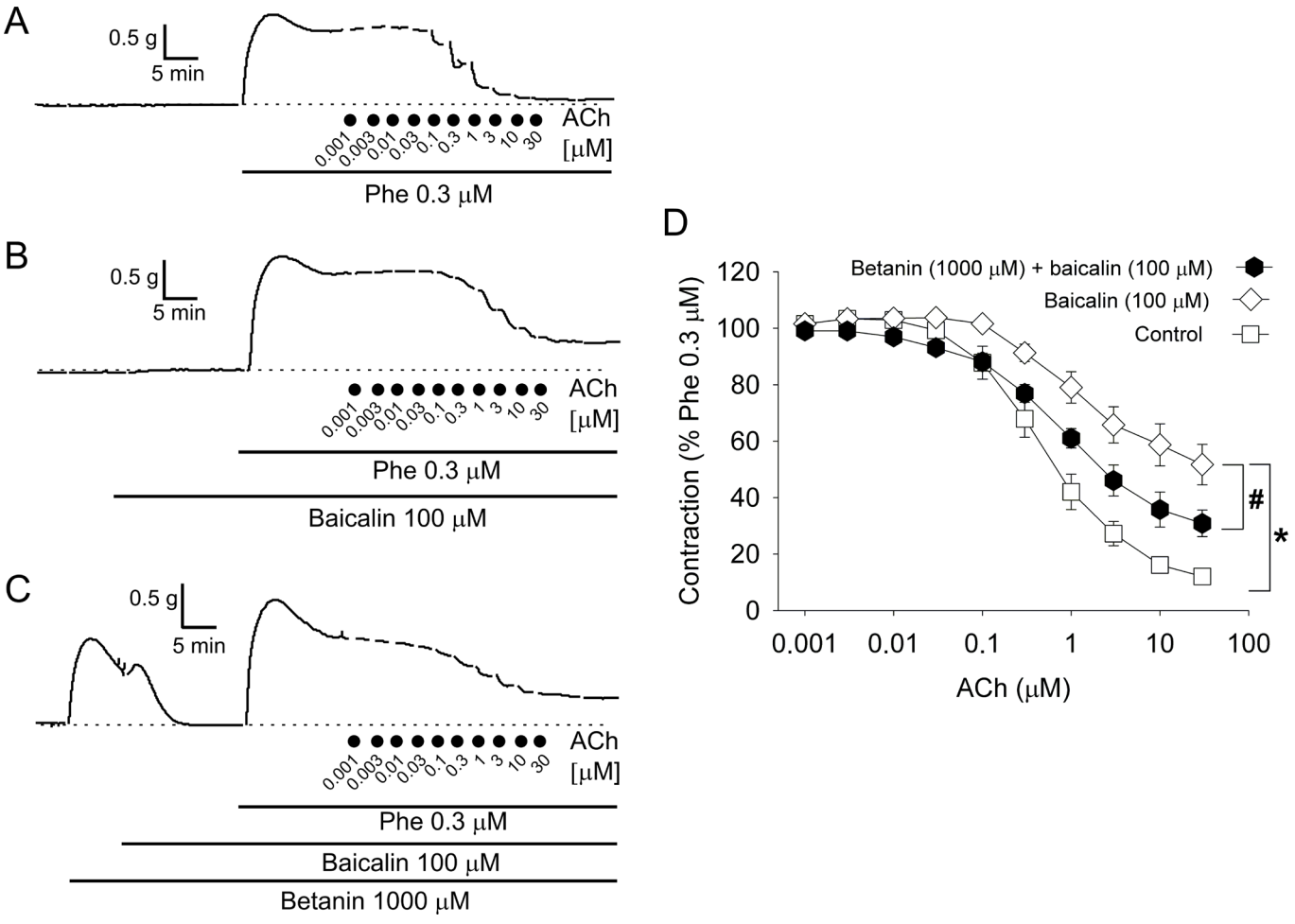

FIGURE 4 - Betanin's influence on endothelial function.

Panels A-C depict representative experiments of endothelium-dependent relaxation by increasing acetylcholine (ACh; 0.001 – 100 μM) in endothelium-intact aortic rings pre-contracted with phenylephrine (0.3 μM). Aortic rings were maintained under different conditions: no additional treatment (control), baicalin (100 μM, a nitric oxide-cGMP-protein kinase G pathway inhibitor), or a combination of baicalin and betanin (1000 μM). Panel D shows vasorelaxant responses expressed as a percentage of the phenylephrine-induced contraction before ACh addition. Data represent mean ± standard error of the mean (SEM). Statistical analysis (two-way ANOVA followed by Holm-Sidak test) compared each treatment group to the control curve, with # and * indicating significance ($p < 0.05$).

In a study testing betanin in vivo in rats, Krantz, Monier, and Wahlström (1980) showed that intravenous administration transiently increased both blood pressure and heart rate. In vitro, they also showed that betanin increased the magnitude of the rhythmic contractions in isolated preparations of rat portal vein. These effects were partially reversed by phentolamine, a non-selective blocker of alpha-adrenergic receptors (Morton *et al.*, 2007), suggesting a potential involvement of adrenergic receptors in the effects of betanin.

In the present study, we investigated the effects of betanin on isolated preparations of rat aorta maintained under resting tension. The addition of betanin to the bath caused concentration-dependent contractions in the isolated aortic preparations within a micromolar concentration range. This potent contractile effect of betanin was eliminated in the presence of prazosin, a selective alpha-1 adrenergic receptor blocker that primarily targets peripheral alpha-1 receptors rather than alpha-2 adrenergic receptors (Ford *et al.*, 1997). These findings support the hypothesis that alpha-adrenergic receptors are involved in the contractile effects of betanin. The lack of effect on betanin-induced contractions in aortic preparations pretreated with guanethidine or yohimbine suggests that betanin's action does not depend on, respectively, the release of norepinephrine from sympathetic nerve endings or the activation of alpha-2 adrenergic receptors (Day, 1962; Bylund *et al.*, 1992).

To investigate the dependence of the contractile effect caused by betanin on extracellular Ca^{2+}, a set of experiments were conducted using aortic preparations maintained in a Ca^{2+}-free medium. Under these conditions, no contractions were observed upon the addition of increasing concentrations of betanin, suggesting that betanin induces Ca^{2+} influx from the extracellular space. An important contributor to smooth muscle contraction is the recruitment of Ca^{2+} from the extracellular medium through voltage-gated Ca^{2+} channels (Somlyo, Somlyo, 1968). To assess this, we tested the effects of betanin in the presence of verapamil, an L-type voltage-operated Ca^{2+} channel blocker (Namba, Tsuchida, 1996). Thus, in aortic preparations maintained in Ca^{2+}-containing MKHS, the previous treatment with verapamil significantly reduced the vasoconstrictive effects induced by increasing concentrations of betanin. These findings suggest that, at least partially, betanin promoted its vasoconstrictive effects by inducing transmembrane Ca^{2+} influx through voltage-dependent channels.

In blood vessels, the endothelial layer regulates vascular tone through the release of both vasoconstrictors and vasodilators (Rubanyi *et al.*, 1990). We investigated whether the endothelial layer might interfere with the contractile effects induced by betanin in rat aorta. This was assessed using endothelium-denuded preparations, a condition previously confirmed by the lack of relaxation after challenge with acetylcholine. Under these experimental conditions, a significant leftward shift was observed in the concentration-response curve of rat aorta to increasing concentrations of betanin. In fact, both the EC_{50} and the maximal effect of the contraction induced by betanin were significantly higher in endothelium-denuded preparations compared to the respective values in endothelium-intact aortic rings. These findings suggest a potential inhibitory influence of the endothelial layer on the contractile effects induced by betanin. It is known that the NO-cyclic GMP pathway accounts for most of the inhibitory stimuli derived from endothelium on alpha-adrenergic responses in aortic rings (Topouzis, Schott, Stoclet, 1991).

To further investigate the endothelial influence on the effects of betanin, we studied aortic preparations previously treated with baicalin, an inhibitor of the NO-cyclic GMP pathway (Huang *et al.*, 2004). The inhibitory effect of baicalin on endothelial function was demonstrated by a significant decrease in the vasorelaxant response of endothelium-intact aortic rings to acetylcholine, which was added at the peak of a contraction induced by phenylephrine. Interestingly, in aortic rings pretreated with both baicalin and betanin, the relaxant response to acetylcholine was less impaired and achieved higher values compared to the response observed in aortic rings treated with baicalin alone. These findings suggest that in aortic rings with intact endothelium, betanin induces a weaker contraction compared to preparations without functional endothelium, possibly due to its ability to enhance the formation and/or release of endothelial relaxing factors. Supporting this notion, oral administration of betanin has shown a protective effect against oxidative stress in a peripheral

artery vasospasm model in rats. This suggests that betanin can prevent and reduce vascular maladaptive pathological changes caused by a sustained vasospasm (Tural *et al*., 2021).

While the vasorelaxation induced by beetroot is primarily attributed to the increased blood levels of nitrite and nitrate, it is important to consider that the biological activity of whole plant extracts may result from the combined effects of several compounds (Raskin, Ripoll, 2004). Synergy, enhanced bioavailability, cumulative effects, or additive properties of constituents can explain the different activity observed between whole plant extracts and their isolated compounds (Carmona, Pereira, 2013). Therefore, even though betanin causes acute vasoconstriction, it could still contribute to the long-term cardioprotective effects associated with beetroot consumption. This contribution could be due to betanin's modulatory effect on endothelial function, which includes its ability to reduce the formation of reactive oxygen species (ROS) and the expression of inflammatory cytokines and genes encoding oxidant enzymes (Gentile *et al*., 2004; Krajka-Kuźniak *et al*., 2013; Esatbeyoglu *et al*., 2015; da Silva *et al*., 2019).

While this study focused on the direct effects of betanin on vascular smooth muscle, some studies propose that betanin may undergo metabolism to generate metabolites. We lack evidence regarding potential synergistic or opposing effects of these metabolites with betanin in the vasculature. Interestingly, Muramatsu *et al.* (2023) demonstrated that a significant portion of the antioxidant capacity in red beetroot juice and betanin persists even after heat degradation of betanin. Notably, studies have reported the antioxidant capacity of betanin degradation products, such as betalamic acid and cyclo-dopa derivatives (Gandía-Herrero, Escribano, García-Carmona, 2012; Nakagawa *et al*., 2018). Therefore, the metabolites generated from betanin degradation likely exert a synergistic effect on the vasculature, potentially contributing to the cardioprotective effects of beetroot consumption. This warrants further investigation.

The demonstrated pharmacological profile of betanin suggests potential practical applications, although this aspect requires further investigation. Compounds that induce vasoconstriction through activation of alpha-adrenergic receptors are considered pharmacologically relevant for treating conditions like hypotension (Hollenberg, 2011) or nasal congestion (Laccourreye *et al*., 2015). Furthermore, chronic betanin consumption might improve pathophysiological events caused by oxidative stress and inflammation, leading to cardiovascular disease.

In conclusion, the present study demonstrated that betanin exerted vasoconstrictive effects in isolated aortic rings, which were attenuated by an alpha-1 adrenergic antagonist and appeared to depend on transmembrane Ca^{2+} influx. Additionally, our findings highlight the significant modulatory role of the endothelium in the vasoconstrictive effects induced by betanin. Collectively, these results suggest that betanin is a substance of potential interest in the context of vascular functions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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