The relaxing effect of *Poncirus fructus* and its flavonoid content on porcine coronary artery

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Coronary artery disease is a common occurrence in human, and causes enormous social cost. *Poncirus fructus* (PF), the dried immature fruits of *Poncirus trifoliata Rafinesquem*, is used in the treatment of womb contraction and dyspepsia, as a prokinetic, and in improving blood circulation. This study was performed to investigate the effects of PF and some of its flavonoids components on the coronary from the pig. The arterial ring was suspended by a pair of stainless steel stirrups in an organ bath. The end of the upper stirrup was connected to an isometric force transducer. A dose-dependent induction of relaxation was observed by both water and 70% ethanol extracts of PF in the porcine coronary artery pre-contracted with U46619 (100 nM), a stable analogue of the potent vasoconstrictor thromboxane A_2. The 70% ethanol extract showed more efficacy than the water extract. Pretreatment of the artery with L-NAME (100 μM), a nitric oxide synthase inhibitor, resulted in a significant reduction in the relaxation induced by PF extract. In addition, ODQ (10 μM), a soluble guanylate cyclase inhibitor, also significantly reduced the effects of PF extracts. Hesperidin, a flavonoid present in PF, induced very weak relaxation of the porcine coronary artery at a high concentration (100 μM), while its aglycone, hesperetin, demonstrated a dose-dependent relaxation. In conclusion, PF extracts induced relaxation in the porcine coronary artery, partially through the nitric oxide-cGMP pathway, and the aglycones of flavonoids might be also involved in the relaxation of the same artery.

Keywords: Porcine coronary artery, *Poncirus fructus*, hesperidin, hesperetin

Coronary arteries supply oxygen-rich blood to the heart muscles (myocardium). Limited arterial function results in coronary artery disease (CAD). The incidence of CAD is increasing, with many people being afflicted with chronic infections. The results of a study conducted on 1,000 participants in North America reveal that the average incidence rate of coronary artery disease (CAD) is 12.5 in Caucasian men and 10.6 in African-American men [1]. In addition, according to the heart disease and stroke statistics of the American Heart Association, 770,000 Americans suffered from first-time coronary attacks while 430,000 suffered from a repeat attack during 2008. Every year, 190,000 people were newly afflicted with additional silent acute myocardial infarction [2].

Poncirus fructus (PF) is the dried, immature fruit of the *Poncirus trifoliata* Rafinesque. PF grows naturally in the Jeju Island or the Gaduk Island in Korea, and is cultivated in a village in the southern boundaries of the central region. Traditionally, it is used as a prokinetic, and to treat womb contraction, dyspepsia, and improve blood circulation [3]. PF is believed to possess many functional properties: anti-inflammatory [4], anti-allergenic [5], lipid-lowering activity [6,7], inhibition of melanogenesis

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[8], and suppression of body weight gain [9]. A recent study has reported that the lipid-soluble fraction of the PF extract inhibits small intestinal motility, whereas the water extract fraction caused an increase in the small intestine activity *in vitro* and in the distal colon activity *in vivo* in guinea pigs [10]. In addition, it has been reported that the water extract of the PF accelerates the transport of intestinal contents [11], while the hexane extract stimulates rat distal colon [12]. The extracts of PF have been shown to induce relaxation in rat gastric fundus [13]. However, the effects of Poncirus fructus on the arterial vessel have not yet been elucidated.

Flavonoids are secondary metabolites in plants which express diverse bioactivities. Hesperidin, neohesperidin, naringin, and poncirin are the main component flavonoids of the PF [14]. Many flavonoids are known to exert a relaxing effect on the arterial smooth muscle [15-20]. Hesperidin has been reported to produce an anti-hypertensive effect in spontaneously hypertensive rats (SHR). However, the effects of these flavonoids, which are biocomponents of PF, on the coronary artery have been scarcely reported.

The major aim of this study was to investigate the possible relaxing effects of PF on porcine coronary artery. In addition, we attempted to determine if the flavonoids contained in PF [21,22] were responsible for this relaxing action.

Materials and Methods

Materials

PF was purchased from an oriental drug store in Yangnyeong market (Daegu, Korea). A sample (100 g) of PF was boiled in 1 L of distilled water at 100ºC for 2 hours. The water extract was filtered and concentrated *in vacuo*. Finally, the powder was obtained by lyophilization. The 70% ethanol extract was also obtained using the same method.

Three flavonoids that are abundant in PF were used in this study: poncirin, hesperetin, and hesperidin. Hesperetin is an aglycone of hesperidin. All flavonoids were purchased from Sigma-Aldrich (St. Louis, MO, USA).

In order to determine the mechanism of PF action, the following compounds were used: 9,11-dideoxy-9a,11a-methanoepoxy-prostaglandin F2a (U46619, Cayman, Ann Arbor, MI, USA), a stable analog of thromboxane A_2; Nitro-L-arginine methyl ester (L-NAME; Sigma-

Aldrich), a nitric oxide synthase inhibitor; and 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Calbiochem-Novabiochem, La jolla, CA, USA), a soluble guanylate cyclase inhibitor. ODQ was prepared in dimethyl sulfoxide, while the other compounds were diluted in distilled water. In order to obtain the desired concent-rations, physiological salt solutions (PSS) were used.

Animals and tissue preparation

The hearts were obtained from twenty-four landrace pigs (3 months) in the local abattoir and transported to the laboratory in cool oxygenated Krebs solution (physiololical salt solutions; PSS) within 30 minutes. The Krebs solution was prepared with NaCl (118 g), KCl (4.7 g), $MgCl_2$ (1.2 g), $CaCl_2$ (2.5 g), KH_2PO_4 (1.2 g), $NaHCO_3$ (25.0 g), and glucose (10.0 g). The distal portion of the left anterior descending coronary artery (outer diameter, 1-2 mm) was dissected from the heart in PSS. The fat and connective tissues were removed from the coronary artery under the dissecting microscope. The coronary artery was cut into rings (5 mm length). Care was taken to preserve the endothelium (no scratches). The Institutional Animal Care and Use Committee of Kyungpook National University approved the protocols for the animal study (KNU 2012-8).

Measurement of isometric tension

The prepared tissue was suspended by two stainless steel stirrups inan organ bath (volume 10 mL) maintained at 37ºC. The solution was aerated with 95% O_2 and 5% CO_2. The end of the upper stirrup was connected to the transducer (FT-03; Grass-Telefactor, West Warwick, RI, USA). The transducer signal was processed using the Powerlab data acquisition software supplemented with Labchart (v.7.0, ADInstruments, Castlehill, Australia). In order to measure the relaxation ability, PF extracts and other drugs were administered after the contraction reached a plateau level when supplemented with U46619 (100 nM), a stable analogue of thromboxane A_2, a potent vasoconstrictor.

Statistical analysis

All values are expressed as mean±standard error of mean. The statistical significance was determined using the Student's t-test. A *P*-value <0.05 was considered to have statistical significance.
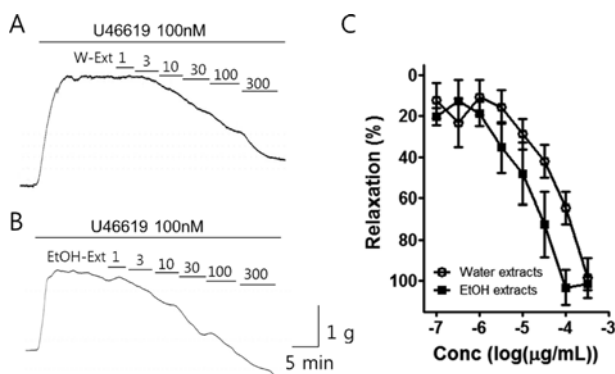
Figure 1. Effect of *Poncirus fructus* water and 70% ethanol extracts on porcine coronary artery. The PF (A) water and (B) 70% ethanol extract induced dose-dependent relaxation in the porcine coronary artery. (C) The 70% ethanol extract was show to be more potent than the water extract (n=8).

Results

Effect of the PF water and 70% ethanol extracts on porcine coronary artery

The porcine coronary artery was contracted by U46619 (100 nM). When this reached a plateau, the water and 70% ethanol extracts were cumulatively administered into the bath (1-300 μg/mL) (Figure 1). The percentage of relaxation effected by the water extract on the porcine coronary artery was determined to be 13.02±7.97, 16.82±7.18, 30.55±7.77, 42.14±7.80, 66.94±8.11, and 98.57±9.84 at doses 1, 3, 10, 30, 100, and 300 μg/mL, respectively. The percentage of relaxation effected by the 70% ethanol extract on the porcine coronary artery was 18.93±5.75, 35.31±12.47, 47.74± 13.0, 72.31±15.10, 98.64±8.82, and 101.33±3.53 at doses 1, 3, 10, 30, 100, and 300 μg/mL, respectively. The potency of the 70% ethanol extract was greater than that of the water extract.

Effects of L-NAME on the effect of PF water extract on porcine coronary artery

The water extract of PF (100 μg/mL) was observed to induce relaxation in the porcine coronary artery. When L-NAME (100 μM), a nitric oxide synthase inhibitor, was administered to this experimental setup, was observed a significant reduction in the relaxation state of the artery (Figure 2). The percentage of relaxation effected by the PF water extract 100 μg/mL on the porcine coronary artery was determined to be 52.79± 7.34. The administration of L-NAME to the experimental setup resulted in a decrease in the percentage of
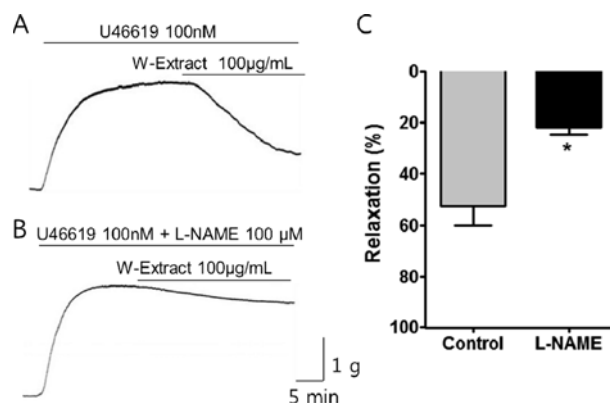

Figure 2. Effect of L-NAME on the relaxation activity of PF water extract on porcine coronary artery. Raw tracings expressing the effect of PF water extract on porcine coronary artery in the (A) absence or (B) presence of L-NAME, a nitric oxide synthase inhibitor. (C) In the presence of L-NAME, the effect of the water extract was significantly reduced. Mean±SEM, *P<0.05, n=5.
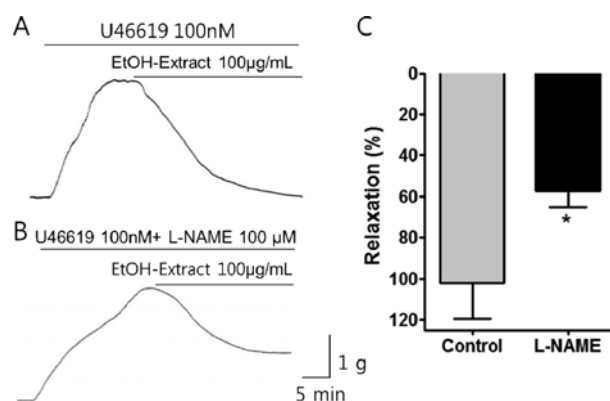

Figure 3. Effect of L-NAME on the relaxation activity of PF ethanol extract on porcine coronary artery. Raw tracings displaying the effects PF of ethanol extract on porcine coronary artery in the (A) absence or (B) presence of L-NAME. (C) L-NAME significantly inhibited the effect of the 70% ethanol extract. Mean±SEM, *P<0.05, n=5.

relaxation of water extract (100 μg/mL)-treated porcine coronary artery to 21.03±5.92.

Effects of L-NAME on the activity of PF ethanol extract on porcine coronary artery

Administration of the PF ethanol extract (100 μg/mL) induced relaxation in the porcine coronary artery. L-NAME (100 μM), a nitric oxide synthase inhibitor, caused a significant reduction in the PF ethanol extract-induced relaxation (Figure 3). The percentage of relaxation induced by PF ethanol extract (100 μg/mL) on the porcine coronary artery before and after administration of L-NAME was 101.86±17.28 and 58.59±21.93, respectively.
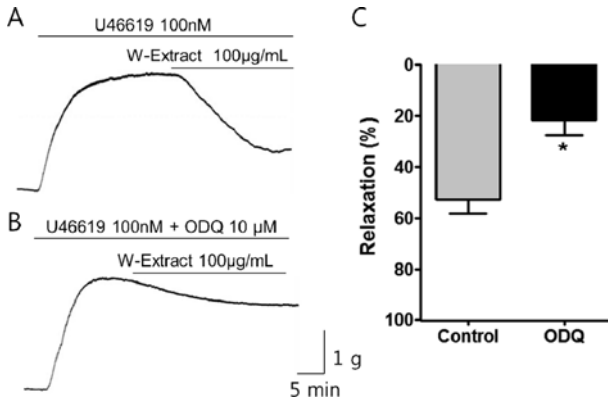
Figure 4. Effect of ODQ on the relaxation activity of PF water extract on porcine coronary artery. Raw tracings showing the effects of PF water extract on porcine coronary artery in the (A) absence or (B) presence of ODQ, a guanylate cyclase inhibitor. (C) In the presence of ODQ, the effect of the water extract was observed to be significantly reduced. Mean±SEM, *P<0.05, n=5.
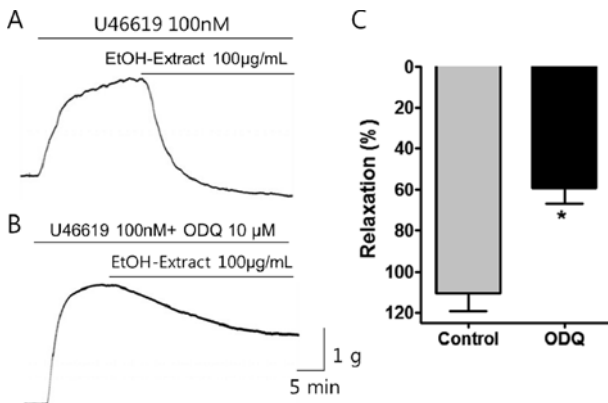

Figure 5. Effect of ODQ on the relaxation activity of the PF ethanol extract on porcine coronary artery. Raw tracings demonstrating the effects of PF ethanol extract on porcine coronary artery in the (A) absence or (B) presence of ODQ. (C) In the presence of ODQ, the effect of the 70% ethanol extract was observed to be significantly reduced. Mean±SEM, *P<0.05, n=5.

Effects of ODQ on PF water extract activity on porcine coronary artery

The administration of ODQ (10 µM), a guanylate cyclase inhibitor, induced a significant reduction in the PF water extract-induced relaxation of porcine artery (Figure 4). The percentage of relaxation in porcine coronary artery subjected to PF water extract (100 µg/mL) before and after ODQ administration was determined to be 55.79±7.34 and 22.49±6.30, respectively.

Effect of ODQ administration on PF ethanol extract-treated porcine coronary artery

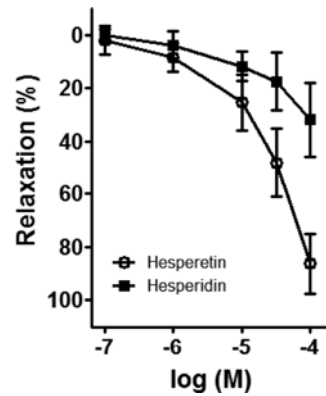The administration of ODQ (10 µM) resulted in a

Figure 6. Effects of hesperidin and hesperetin on porcine coronary artery. The relaxation potency of hesperetin, an aglycone of hesperidin, was greater than that of hesperidin in the porcine coronary artery. Mean±SE, n=5.
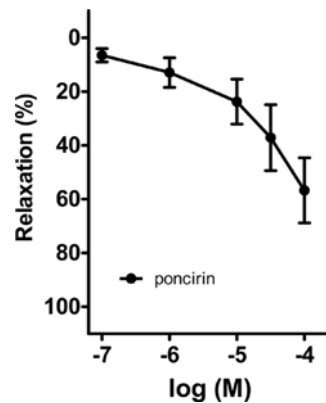

Figure 7. Effect of poncirin on porcine coronary artery. Poncirin-induced relaxation in the porcine coronary artery was pre-contracted by U46619, in a dose-dependent manner. Mean±SE, n=5.

significant reduction in the PF ethanol extract-treated porcine artery (Figure 5). The percentage of relaxation seen in porcine artery treated with PF ethanol extract (100 µg/mL) before and after ODQ administration was 105.86±17.28 and 59.25±19.69, respectively.

Effect of hesperidin and hesperetin on porcine coronary artery

Hesperidin and hesperetin are known flavonoids present in PF. These were administered into the bath via a cumulative method (0.1-100 µg/mL) (Figure 6). The percentage of relaxation effected by hesperidin on the porcine coronary artery was determined to be 3.08± 1.00, 5.46±1.35, 9.74±6.28, 14.00±3.18, 18.81±4.62, and 30.64±7.35, at doses of 0.1, 1, 3, 10, 30, and 100 µM, respectively. The percentage of relaxation of the procine coronary artery induced by hesperetin at

doses of 0.1, 1, 3, 10, 30, and 100 μM was determined to be 8.34±1.98, 13.86±3.13, 17.69±9.53, 25.91±13.62, 49.03±13.19, and 82.67±14.77, respectively. The potency of hesperetin was determined to be greater than that of hesperidin.

Effect of poncirin on the porcine coronary artery

Poncirin, a flavonoid present in PF, was administered into the bath in a cumulative method (0.1-100 μg/mL) (Figure 7). The percentage of relaxation effected by poncirin at doses of 0.1, 1, 10, 30, and 100 μM, on the porcine coronary artery was determined to be 7.50±2.42, 18.59±5.56, 26.98±8.67, 41.86±12.46, and 57.70±12.02, respectively.

Discussion

Coronary artery is composed of two main branches, the left and right coronary artery. Both vessels rest in the epicardial fat. The left and right coronary arteries are branched into four principal sections: the right coronary artery, left main coronary artery, left anterior descending artery, and left circumflex artery [23]. The vessels supply oxygen-rich blood to the heart muscles; therefore, inappropriate dilation in these vessels can result in angina pectoris or myocardial infarction [24]. Treatment of unstable angina pectoris with nitrates results in improved exercise tolerance, delaying the time to onset of angina [25].

Polyphenols include flavonoids and tannins. Flavonoids are plant pigments which consist of two aromatic rings and an oxygen heterocycle. According to the configuration and state oxidation of the central heterocycle unit, the flavonoids are classed into eight different types: flavones, flavonols, flavonones, chalcones, isoflavones, anthocyanidins, aurones, biflavones, and neoflavonoids. Hesperetin, which is abundantly found in PF, belong to the flavonone group [26].

In recent times, flavonoids have been investigated in the treatment of ischaemic stroke and in reducing chronic vascular disease (CVD) mortality [26-28]. Flavonoids are known to possess cardio-protective effects [27,28]. Several studies have demonstrated the effect of dietary flavonoids and polyphenols on human health and disease. In the cardiovascular system, flavonoids influence the endogenous regulation of cholesterol synthesis and are known to scavenge free radicals. They have also been discovered to promote endothelial-derived relaxation

factor-induced relaxation in rabbit arteries *in vitro* [29].

In this study, both the water extract and 70% ethanol extract of PF was observed to induce relaxation in the porcine left descending coronary artery in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) of 70% ethanol extract was approximately 10 μg/mL, and that of water extract was approximately 60 μg/mL. The 70% ethanol extract demonstrated approximately six times greater efficacy than the water extract. This difference between the activities of 70% ethanol and water extract has not been previously reported.

In order to identify the mechanism by which the PF extract induces relaxation, the nitric oxide-cyclic GMP pathway, which was representative of the relaxation mechanism in blood vessels, was tested. The nitric oxide synthase induced nitric oxide synthesis via L-arginine in endothelial cells. The nitric oxide, which migrates into the smooth muscle cell, was observed to bind to and activate guanylyl cyclase. This enzyme catalyzes the dephosphorylation of guanosine triphosphate to cyclic guanosine monophosphate, which relaxes the smooth muscle of the blood vessels.

In this study, the relaxation of porcine artery induced by PF extract was observed to be attenuated by L-NAME and ODQ. Both chemicals were observed to suppress the water and 70% ethanol extract-induced relaxation of coronary arteries. However, the relaxation was not completely suppressed, and was only approximately 50% compared to that effected in the control. These results suggested that the relaxing effect of PF extract was at least partially mediated by the nitric oxide-cGMP pathway. However, the endothelium-dependent mechanism of blood vessel relaxation, regulated by the endothelium-derived hyperpolarizing factor [30,31], could not be completely dismissed. It has been reported that the vasorelaxation effected by dietary flavonoids was mediated by the nitric oxide-cyclic GMP pathway as well as the endothelium-derived hyperpolarizing factor [32].

Hesperidin induced very little relaxation in the porcine coronary artery. However, hesperetin, an aglycone of hesperidin, clearly induced relaxation in the porcine coronary artery. It has been hypothesized that flavonoids, which are large and highly polar molecules, could not be absorbed immediately when ingested via the oral route. The flavonoids are hydrolyzed to aglycones by bacterial enzymes in the lower section of the intestine [33,34], and only then might be partially absorbed or may undergo

further biotransformation [35].

Hesperidin is a flavanonol that structurally lacks the C(2)-C(3) double bond in the C-ring. This compound only slightly affects vascular relaxation. Planar flavonoid structures are believed to express potent vasorelaxation effects [36]. The relationship between the structure and activity of flavonoids and their aglycones have been previously reported in antioxidant research [37]; however, their activities in coronary arteries have not been elucidated. In this study, we discovered that the aglycones of PF flavonoids may be involved in effecting relaxation in the porcine coronary artery.

Poncirin, which is present abundantly in PF, was also observed to induce relaxation in the porcine coronary artery. Poncirin, like hesperidin, is also a flavonone and lacks the C(2)-C(3) double bond. However, in this study, this compound effected mild vasodilation in the porcine coronary artery. However, the effect of poncirin on the vessels has not been reported.

The vasorelaxation effects of flavonoids and its mechanism have been reported to be closely associated with the endothelium-derived nitiric oxide pathway [32] and the cyclic adenosine monophosphate/protein kinase A-dependent pathway, and to be partially mediated by a potassium channel, such as the BKca [36]. The anti-hypertensive effect of flavonoids has also been reported [38], and its ability to induce vasorelaxtion has been associated with the design in chemical structure [36].

In conclusion, the PF extracts have been determined to induce relaxation in the porcine coronary artery. These effects were at least partially induced by the PF flavonoids. The relaxation is partially mediated by the NO-cGMP pathway. The relaxation effects of other pathways, such as the cAMP pathway and the endothelium-derived hyperpolarizing factor, must be investigated in the future. Finally, the aglycones of PF flavonoids could be involved in effecting relaxation in the porcine coronary artery, indicating that the bioactive forms of flavonoids express greater efficacy.

Acknowledgments

Conflict of interests   The authors declare that there is no conflict of interests.

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