Insulin Secretion by Bioactive Anthocyanins and Anthocyanidins Present in Fruits

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Anthocyanins are responsible for a variety of bright colors including red, blue, and purple in fruits, vegetables, and flowers and are consumed as dietary polyphenols. Anthocyanin-containing fruits are implicated in a decrease in coronary heart disease and are used in antidiabetic preparations. In the present study, we have determined the ability of anthocyanins, cyanidin-3-glucoside (**1**), delphinidin-3-glucoside (**2**), cyanidin-3-galactoside (**3**), and pelargonidin-3-galactoside (**4**), and anthocyanidins, cyanidin (**5**), delphinidin (**6**), pelargonidin (**7**), malvidin (**8**), and petunidin (**9**), to stimulate insulin secretion from rodent pancreatic β-cells (INS-1 832/13) in vitro. The compounds were tested in the presence of 4 and 10 mM glucose concentrations. Our results indicated that **1** and **2** were the most effective insulin secretagogues among the anthocyanins and anthocyanidins tested at 4 and 10 mM glucose concentrations. Pelargonidin-3-galactoside is one of the major anthocyanins, and its aglycone, pelargonidin, caused a 1.4-fold increase in insulin secretion at 4 mM glucose concentration. The rest of the anthocyanins and anthocyanidins tested in our assay had only marginal effects on insulin at 4 and 10 mM glucose concentrations.

KEYWORDS: Anthocyanins; anthocyanidins; pancreatic β-cells; insulin secretion; type-2 diabetes

INTRODUCTION

The function of insulin is to maintain normal blood glucose levels either by suppression of glucose output from liver or by the stimulation of glucose uptake and its metabolism (*1*). Insufficient release of insulin or loss of insulin action at target tissues causes aberrant glucose and lipid metabolism. This results in elevated glucose levels in the blood, a hallmark of diabetes (*2*). There are two types of diabetes, type-1 (insulin-dependent diabetes) and type-2 diabetes (noninsulin-dependent diabetes). Type-1 diabetes results from autoimmune destruction of pancreatic β-cells, the cells that secrete insulin, which leads into insulin insufficiency. Type-2 diabetes is more prevalent and is caused by the inability of β-cells to secrete sufficient amounts of insulin to overcome insulin resistance established by genetic and environmental factors (*3*). Insulin resistance is a disorder in which insulin inadequately stimulates glucose transport in skeletal muscle and fat and inadequately suppresses hepatic glucose production. The mechanisms involved that prevent the β-cell from secreting sufficient amounts of insulin to overcome peripheral insulin resistance remain to be established. Oral hypoglycemic agents that directly stimulate insulin release from β-cells (e.g., sulfonylurea-based drugs), however, have shown

that insulin secretion from islets of type-2 diabetic patients can be elevated sufficiently to overcome peripheral insulin resistance and normalize blood glucose levels. One of the disadvantages of using sulfonylurea-based drugs is that it fails to control normal blood glucose levels (*4*). These drugs also adversely affect the ability of β-cells to secrete insulin and cause weight gain (*4*). Hence, there is a role for dietary constituents that can regulate blood glucose levels or induce insulin production by pancreatic β-cells.

The consumption of a diet low in fat and rich in antioxidants reduces the risk of obesity and insulin resistance (*5*). Anthocyanins belong to antioxidant polyphenols and are present in various foods and beverages. Consumption of anthocyanins is associated with a reduced risk of several degenerative diseases such as atherosclerosis, cardiovascular disease, cancer, and diabetes (*6*). These compounds are well-known free radical scavengers and are reported as potential chemopreventive agents (*7*). For example, the serum antioxidant capacity was increased by the consumption of strawberries, cherries, and red wine (*8*–*10*). Recent studies demonstrated that the anthocyanin, cyanidin 3-glucoside, reduced the high fat diet-induced obesity in mice (*11*). Therefore, the natural colorants present in food have attracted consumers due to their safety and nutritional and therapeutic values (*12*). Because anthocyanins are widely consumed, additional biological activities of these compounds will be of great interest.

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The fruits of the *Cornus* species are a rich source of anthocyanins. The fruits of *Cornus mas* L., also known as the European and Asiatic Cornelian cherry, are used in the preparation of beverages in Europe (*13*). In traditional medicine, *Cornus officinalis* fruits are known for their analgesic and diuretic activities (*14*). The *Cornus* fruits are also one of the major constituents of several antidiabetic herbal preparations in Asian countries (*15*). Our earlier investigation of the fruits of *C. mas* and *C. officinalis* revealed that both contained high levels of anthocyanins (*16*). Therefore, we hypothesized that anthocyanins and anthocyanidins may be responsible for the anecdotal antidiabetic activity reported for *Cornus* fruits. In this paper, we report glucose-induced insulin release by anthocyanins and anthocyanidins by pancreatic β-cells. This is the first report of anthocyanins and anthocyanidins as insulin secretagogues.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from Invitrogen (Grand Island, NY). All organic solvents used were ACS reagent grade. HEPES, penicillin—streptomycin, glutamine, sodium pyruvate, 2-mercaptoethanol, trypsin-ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA; RIA Grade), Folin—Ciolatues reagent and chemicals used for the preparation of buffers were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The anthocyanidins, cyanidin, delphinidin, pelargonidin, malvidin, and petunidin, used in the assay were purchased from Chromadex (Laguna Hills, CA).

Anthocyanins. Delphinidin-3-glucoside (**2**) was purified from *C. officinalis* fruits. Cyanidin-3-galactoside (**3**) and pelargonidin-3-galactoside (**4**) were isolated from *C. mas* fruits. Pure cyanidin-3-glucoside (**1**) used in this study was from our storage at $-20\ ^\circ$C.

Isolation and Purification of Anthocyanins. The *Cornus* fruits were blended with water (pH 3) and filtered. The filtrate was passed through XAD-16 Amberlite resin in a column, and the resin with the adsorbed anthocyanins was washed repeatedly with water (*17*). The XAD-16 resin was then eluted with acidic MeOH (pH 3), and the resulting solution was concentrated under reduced pressure to yield a crude anthocyanin fraction. This fraction was purified by medium pressure liquid chromatography (MPLC) column (C_{18} silica) using MeOH:H_2O (pH 3) under gradient conditions. The anthocyanins were eluted with a MeOH:H_2O (65:35, v/v) solvent system. The purity of the compounds was checked by high-performance liquid chromatography (Waters Corp.) using Capcell C_{18} analytical column under gradient conditions. The solvents used were A, TFA:H_2O (99.9:0.1, v/v), and B, H_2O:CH_3CN:CH_3COOH:TFA (50.4:48.5:1.0:0.1, v/v/v/v). The gradient was $20-60\%$ B in 26 min and to 20% B in 30 min at a flow rate of 0.8 mL/min. The peaks were detected at 520 nm using a photodiode array detector.

Insulin Secretion Studies. INS-1 832/13 cells (kindly provided by Dr. Christopher Newgard, Duke University, NC) (*18*) were routinely cultured in 5% CO_2/air at 37 $^\circ$C in RPMI-1640 medium containing 11.1 mM glucose and supplemented with 10% FBS, 10 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Cells were passed weekly after trypsin-EDTA detachment. For static secretion studies, cells were plated on 24 well plates at a density of 0.64×10^6 cells per well and grown for 24 h. The cells were then cultured for an additional 24 h in RPMI-1640 containing 4 mM glucose and the supplements described above. Cells were then incubated twice for 30 min in Krebs Ringer Bicarbonate buffer (KRBB) containing 4 mM glucose and 0.1% BSA. Cells were rapidly washed with KRBB and incubated for 60 min KRBB containing 4 or 10 mM glucose with or without the indicated anthocyanins or anthocyanidins. The medium was then removed for determining insulin release. The cells were then washed twice with PBS and dissolved in 1 M NaOH. The cellular protein concentration was then determined by the Lowry assay. Anthocyanins and anthocyanidins were dissolved in dimethyl sulfoxide (DMSO) to obtain desired concentrations. The final concentration of DMSO was 0.1%.

The insulin secreted into the medium by the cells was determined by radioimmunoassay (RIA) and normalized to total cellular protein.

RIA. The kit for RIA was purchased from LINCO Research Inc. (St. Charles, MO), and the assay was conducted according to the manufacturer's directions. Briefly, $0.1-10$ ng of insulin standards (100 μL) were added to 12 mm $\times$ 75 mm test tubes. Similarly, samples (25 μL) from the insulin secretion studies were also added to the test tubes. To this, an aliquot (75 μL) of assay buffer was added. The ^{125}I-labeled insulin (100 μL) was then added to each test tube. An aliquot of 100 μL anti rat insulin antibody was added to the tubes, mixed, and incubated at 4 $^\circ$C for 24 h and incubated further with a 1 mL aliquot of the precipitating reagent for 20 min at 4 $^\circ$C to precipitate the insulin bound to the antibody. The tubes were then centrifuged, and the radioactivity was measured using a γ-counter.

Lowry Protein Assay. The amount of protein in the assay wells was determined by the Lowry method (*19*). The Lowry assay solution was prepared by combining the Lowry solution, $CuSO_4$·$5H_2O$ (1%), and sodium tartarate (1%). Briefly, the protein sample (100 μL) and Lowry mixture (1 mL) were mixed in a test tube (12 $\times$ 75). The Folin—Ciolatues reagent (100 μL) was added to these tubes, mixed, and incubated for 30 min at room temperature. The optical density of resulting solutions was read at 700 nm using a UV spectrophotometer.

RESULTS AND DISCUSSION

The *Cornus* fruits are used in antidiabetic traditional Chinese prescription medicines such as "Hachimi-Gan" (*15*). We have recently reported the quantification of anthocyanins in *Cornus* spp. fruits (*16*). Our investigation of *Cornus* fruits indicated that the primary bioactive components in them were cyanidin, delphinidin, and pelargonidin glycosides. Therefore, we have focused our attention on the insulin-secreting ability of these anthocyanins and their aglycones using pancreatic β-cells in order to substantiate the anecdotal use of *Cornus* fruits in antidiabetic preparations. We have also included petunidin, malvidin, and peonidin aglycones in our assay since they are abundant in other fruits.

Anthocyanins are water soluble compounds. The aqueous extracts of *C. mas* fruits contained sugars, bioflavonoids, and anthocyanins and hence were fractionated by XAD-16 resin. The resulting anthocyanin fraction eluted from the resin was purified by MPLC to afford pure anthocyanins. The glucose-induced insulin production by INS-1 832/13 cells was determined at 4, 10, and 16 mM glucose concentrations, and we found that the insulin secretion reached a lag phase at 10 mM glucose concentration (data not presented). The glucose concentration at 4 mM level is representative of the normal glucose level in human (*20*). The insulin secretion per mg of protein by cells at 10 mM glucose was 3-fold higher when compared to the insulin secretion at 4 mM glucose concentration.

We have tested anthocyanins and anthocyanidins (**Figure 1**) at 4 and 10 mM glucose loads in the cell growth medium. Anthocyanins and anthocyanidins were assayed initially at 50 μg/mL concentration. The anthocyanin, **1**, showed an increase in insulin secretion at 4 mM glucose by 9 ng/mg of protein (1.3-fold) whereas it enhanced the insulin secretion by 1.43-fold (119 ng/mg protein) at 10 mM glucose concentration (**Figure 2a**). Compound **2** was the most active anthocyanin tested and showed a 1.8-fold increase (49 ng/mg of protein) in insulin secretion at 4 mM glucose concentration. However, at 10 mM glucose, it exhibited only a 1.4-fold (113 ng) increase (**Figure 2a**) in insulin production. The insulin secreted by cells at 4 and 10 mM glucose concentrations in this assay were 27 and 83 ng of insulin per mg protein, respectively. The anthocyanins, **3** and **4**, did not increase the insulin secretion at 4 mM glucose concentration. However, **3** showed an increase of 17 ng/mg of protein of insulin (1.2-fold) at 10 mM glucose
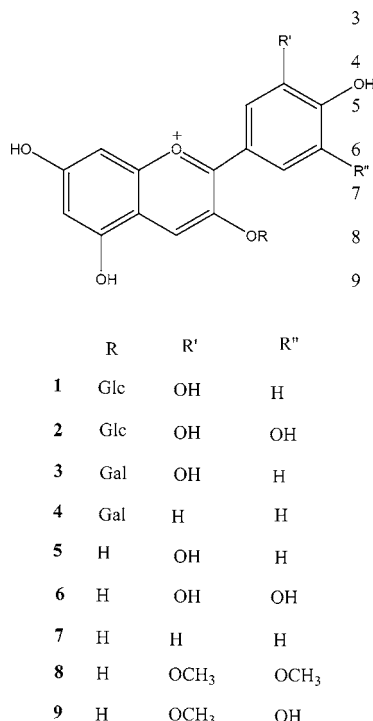
30 *J. Agric. Food Chem.,* Vol. 53, No. 1, 2005

Jayaprakasam et al.

Figure 1. Structures of anthocyanins **1**–**4** and anthocyanidins **5**–**9**.

concentration (**Figure 3**). Compound **4** was tested only once due to the limitation of sample.

The anthocyanin **1** was evaluated for dose-dependent insulin secretion at 5, 10, 50, 100, and 250 μg/mL concentrations. The glucose concentration used in this assay was 4 mM level, which is representative of the normal glucose level in human (*20*). At this concentration, untreated cells secreted 33 ng of insulin/mg of protein. The insulin secreted by **1** treated cells was 46 ng of insulin per mg protein at 5 μg/mL. However, there was no significant difference in insulin secretion at 10, 50, 100, and 250 μg/mL concentrations of compound **1**. We did not have an adequate supply of **2** to conduct dose-dependent assays.

The anthocyanidins were assayed at 50 μg/mL concentration. The aglycone of **1**, cyanidin (**5**), enhanced insulin secretion by 1.5-fold (29 ng/mg of protein) at 4 mM glucose whereas at 10 mM glucose it secreted 88 ng/mg of protein (**Figure 2b**). The untreated cells at 4 and 10 mM glucose secreted 19 and 83 ng insulin/mg of protein, respectively, in this set of assay. The aglycone delphindin (**6**) showed an increase in insulin secretion by 6 ng/mg of protein at 4 mM glucose concentration and was not significant. Delphinidin did not show glucose-induced insulin secretion at 10 mM glucose (**Figure 2b**). Pelargonidin was the most active anthocyanidin, and it secreted 49 (1.4-fold) and 91 (1.2-fold) ng of insulin/mg of protein at 4 and 10 mM glucose, respectively (**Figure 3**). The aglycone petunidin (**9**) increased insulin secretion by 4 ng of insulin/mg protein at 4 mM glucose concentration. However, malvidin (**8**) did not show an increase in insulin secretion with respect to the untreated cells.

Reports indicate that consumption of fruits and vegetables, especially rich in polyphenols, decreased the incidence of type-2 diabetes (*21–23*). Also, it is known that dietary antioxidants protect pancreatic β-cells from glucose-induced oxidative stress. Anthocyanins are abundant in fruits, vegetables, and processed food products such as wine, cider, and tea. However, little is known of its ability to reduce or prevent diabetes. Our results suggested that both anthocyanins and anthocyanidins are insulin secretagogues. The most potent among them was **2**, and it significantly induced the insulin secretion at 4 and 10 mM
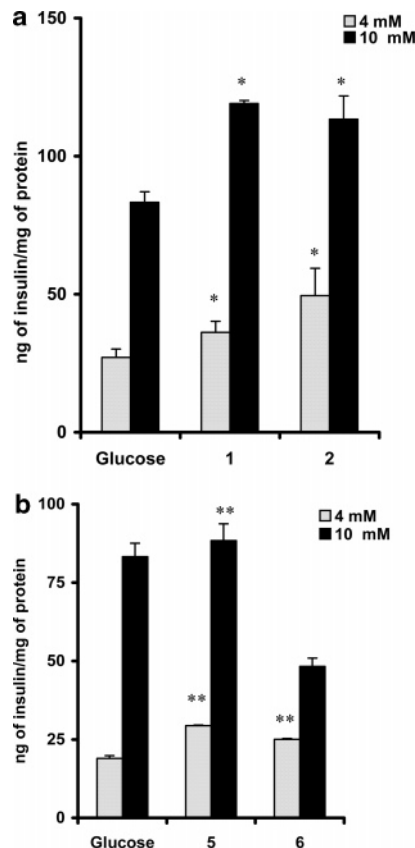

Figure 2. (**a**) Amount of insulin secreted per milligram of protein by compounds **1** and **2** and (**b**) by compounds **5** and **6** in the presence of 4 and 10 mM glucose. The final DMSO concentration in the assay wells was 0.1%. The results represented are the average of three or five independent experiments, and each sample was assayed in duplicate. Insulin secretion by compounds **1**, **2**, **5**, and **6** was significant at * (95% or $p \leq 0.05$) or ** (99% or $p \leq 0.01$) as determined by LSD using the *t*-test.
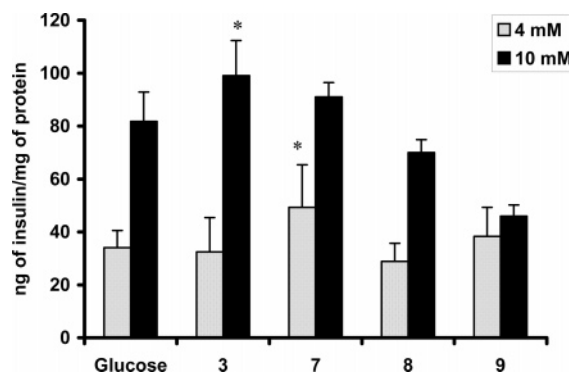

Figure 3. Insulin secreted by compounds **3** and **7**–**9** at 4 and 10 mM glucose concentrations. The amount of insulin secreted was normalized to milligrams of protein. The final DMSO concentration in the assay wells was 0.1%. The results represented are the average of three independent experiments, and each sample was assayed in duplicate. Insulin secretion by compounds **3** and **7**–**9** was significant at * (95% or $p \leq 0.05$) as determined by LSD using the *t*-test.

glucose concentrations as compared to the untreated cells. Although **1** was less active than **2** at a lower glucose concentration, it was more active at a higher glucose concentration. Among the galactosides, **4** did not induce insulin secretion at 4 and 10 mM glucose concentrations studied whereas **3** showed a significant increase in insulin secretion. The ability of

Insulin Secretion by Anthocyanins and Anthocyanidins

J. Agric. Food Chem., Vol. 53, No. 1, 2005 **31**

anthocyanins studied to secrete insulin was in the increasing order of **2** > **1** > **4**. This indicated that the number of hydroxyl groups in ring B of anthocyanins played an important role in their ability to secrete insulin. Among the anthocyanidins tested, pelargonidin was the most active at 4 mM glucose. Other aglycones did not potentiate significant insulin secretion at 4 or 10 mM glucose concentrations studied.

This is the first report of insulin secretion by anthocyanins and anthocyanidins when exposed to pancreatic β-cells. Our results suggest that *Cornus* fruits, cherries, and berries containing these anthocyanins are useful for the prevention of type-2 diabetes. Also, isolated and purified anthocyanins and anthocyanidins from fruits and vegetables may be useful to treat type-2 diabetes. However, in vivo studies and clinical evaluation of these compounds must be carried out to further validate our in vitro results. Because anthocyanidins and anthocyanins are not toxic to humans, it is important to evaluate their clinical efficacy for potential application in the prevention and treatment of type-2 diabetes.

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