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1. Supplemental Methods

1.1. Materials. Chemicals used in adult rat cardiomyocytes isolation, ET1, HCT, ascorbic acid, alpha-smooth muscle actin (α -SMA) antibody, lactate dehydrogenase, calcium ionophore, and cyclopiazonic acid were obtained from Sigma-Aldrich (Missouri, USA). Dulbecco's Modified Eagle's Medium (DMEM)/F12, Spinner Minimum Essential Medium (SMEM), penicillin, and streptomycin were from Gibco (California, USA). Pyruvate kinase was obtained from Roche Diagnostics (Mannheim, Germany). Collagenase II was from Worthington Biochemical Corporation (Minnesota, USA). Extra domain A (ED-A) fibronectin antibody was from Millipore (Massachusetts, UAS). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Abcam (Massachusetts, USA).

1.2. High-Purity Cyanidin 3-O-glucoside chloride Preparation and Quality Assessment.

Cyanidin-3-*O*-glucoside chloride (C3G) was extracted and purified to >98% from ProC3G powder, a commercially available, highly concentrated black rice extract containing ~30% C3G (w/w) (Chromadex, Irvine, CA). In brief, C3G (and other anthocyanins) were extracted from ProC3G powder, using weakly acidified methanol (0.01% HCl), in a sonic bath at 37°C, for 1 hour. The resulting methanolic extract was vacuum filtered (Whatman No.4), and reduced to near dryness via rotary evaporation (Heidolph, Schwabach, Germany).

The resulting solid was then reconstituted in Milli-Q water (EMD Millipore, Billerica, MA), prior to chromatographic purification via a Büchi X50 flash system (Büchi, Geneva, Switzerland), with separation of anthocyanins attained using 100 gram C18 RediSep RF Gold™ flash columns (15mm x 230mm; column volume 87.7mL), with a 20-40 μ m particle size (Teledyne Isco, Lincoln, NE), as per our previously published method (1). Absorbance at 520 nm was monitored and the C3G peak was isolated from other anthocyanins via robotic fraction collection.

Each C3G fraction was purity checked via UPLC-MS/MS (Waters Canada, Mississauga, ON), and suitably pure fractions were subsequently pooled and dried using rotary evaporation. ¹H NMR spectra in CD₃OD and 5% TFA-D (Cambridge Isotope Laboratories, Andover, MA) were collected using a Bruker Avance III 600 NMR (Bruker Biospin Ltd., Milton, ON), as per previously published methods¹ and were used to assess the purity of C3G batches and to monitor for possible crystallized water or solvent contaminants.

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Additional purity checks relying on empirically calculated molar extinction coefficients (ϵ) were used to detect possible crystallized salt (ionic) contaminants, which would be otherwise undetectable via LC-MS or NMR techniques. Absorbance at 530nm versus concentration (moles/L) was plotted for C3G-chloride dilution series, prepared from individual batches of C3G in 1% HCl methanol, where starting masses were precisely measured using a XP26 microbalance (Metler Toledo, Mississauga, ON), accurate to microgram quantities.

Plotting absorbance versus molar concentration, ϵ can be determined as the slope of the line, according to the Beer-Lambert law:

$$A = \epsilon cl,$$

where A = absorbance; c = concentration (moles/L) using a molecular weight of 484.84 g/mol for cyanidin-3-*O*-glucoside chloride; l = spectrophotometer path length of 1 cm; and ϵ is the molar extinction coefficient.

C3G batches were deemed free from possible crystallized salt contaminants, when empirically determined ϵ were found to lie in the range of literature values of 30200 and 34300 $M^{-1}cm^{-1}$, as reported by Swain, and Siegelman and Hendricks.^{2,3}

Individual batches of C3G that met stringent purity (98+%) requirements were mixed together, homogenized, and subsequently aliquoted into individual 50 or 100mg dosed vials, and stored at $-20^{\circ}C$ until required for gavage. Due to the number of animals used and the duration of the feeding trial, a total of 14 grams of highly purified C3G chloride was prepared for this study.

1.3. Isolation and culture of cardiac fibroblasts from adult rats. Cardiac fibroblasts were isolated from adult male Sprague-Dawley rats (150–200 g). Isolated hearts were perfused with (DMEM)/F12 using the Langendorff perfusion system followed by SMEM. The hearts were then digested for 20 minutes at room temperature with SMEM containing 0.1% w/v collagenase II and minced for 15 minutes in diluted collagenase solution (0.05% w/v). Minced tissue was placed into a 50 mL centrifuge tube and suspended in DMEM-F12 growth medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and 1 μ M ascorbic acid and large tissue pieces were allowed to settle to the bottom of the centrifuge tube. Supernatant was transferred to another 50 mL centrifuge tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and cell pellets were re-suspended in growth medium and plated on to 6-well plates. Cells were incubated for 3-4 hours at $37^{\circ}C$ and 5% CO_2 , then washed 2-3 times

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with phosphate buffered saline (PBS) and maintained in fresh growth medium overnight. The following day, cells were washed 2 more times with PBS and fresh medium was added. Cells were allowed to grow at 37°C and 5% CO₂ for another 24 hours before further experimental procedures. As ET1 has been reported to stimulate cardiac fibroblast proliferation and differentiation into myofibroblasts^{4, 5} fibroblasts were stimulated with ET1 for 48 hours. To assess the potential of C3G in modulating proliferation and differentiation of fibroblasts into myofibroblasts, cells were separated into 4 groups: untreated (control group), cells stimulated with ET1 (10⁻⁷ M), cells treated with 60 µM C3G, and cells co-incubated with ET1 (10⁻⁷M) and 60 µM C3G for 48 hours.

1.4. Blood and tissue collection. At the end of the study period, all rats were weighed and anesthetized with ketamine/xylene injection. Non-fasting (random) blood samples were collected with and without heparin and centrifuged at 2500 rpm to obtain plasma and serum which were stored at -80°C for further assessments. Heart tissues were excised, cleaned, and weighed. The atria and the right ventricle were removed and the left ventricle was weighed. The separated heart tissues were flash frozen in liquid nitrogen and stored at -80°C for further analyses. Tibia length was measured and heart weight and left ventricular weights (both normalized to tibia length) were calculated. Thoracic aortas were excised, loosely connected fibrous tissue cleaned off, cut into rings 5 mm in length, and embedded in optimal cutting temperature (OCT) compound in an upright position, and stored at -80°C for further analyses.

1.5. Histological evaluation of the aorta. Aortic samples embedded in OCT were cut into 8 µm sections (three sections per slide) using a cryostat (Leica Biosystems, Nussloch, Germany). Slides were kept at -80°C until stained. To assess histological changes of the aortic wall, sections were stained with hematoxylin and eosin (H & E) according to the manufacturer's instructions (Sigma-Aldrich (Missouri, USA)). Three aortic sections for each rat were randomly selected and stained. The stained sections were examined with light microscopy (Axioskop 2 mot plus microscope, Carl Zeiss, Oberkochen, Germany). Images were captured using 4 X and 20 X objectives with a digital microscope camera (AxioCamStemi 2000 camera). Image-pro analytical software (Maryland, USA) was used to measure wall thickness, lumen diameter, and wall to lumen ratio. Three images of each stained aortic sections were analyzed. Aortic structure data analysis was carried out in a blinded manner.

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1.6. Assessment of SERCA Activity. The kinetic properties of Ca^{2+} -dependent SERCA ATP hydrolysis (i.e. SERCA activity) were examined using a spectrophotometric assay⁶ modified by Duhamel *et al.*⁷ for use on a plate reader (SPECTRAMax, Molecular Devices, California, USA). Left ventricular tissue homogenates were mixed with 4.25 mL of buffer cocktail containing ATPase buffer, lactate dehydrogenase and pyruvate kinase. To allow calcium to permeate cell membranes, 8.8 μL calcium ionophore was added to the assay buffer. Calcium-dependent activity was then measured by varying the amount of calcium (10-25 μL 10mM CaCl_2) loaded in an Eppendorf tube that contained 250 μL buffer cocktail and left ventricle protein. To determine basal ATPase activity, 2 μL of the SERCA inhibitor cyclopiazonic acid (CPA) was used in one sample, and this basal activity was subtracted from total Ca^{2+} -stimulated ATPase activity, which enabled the calculation of Ca^{2+} -stimulated SERCA activity. After plotting a graph of enzyme activity versus Ca^{2+} concentration, maximal enzyme activity (V_{max}), Ca_{50} and the Hill slope were calculated using GraphPad Prism software. Ca_{50} was defined as the concentration of Ca^{2+} that elicits 50% of V_{max} when graphed using a sigmoidal fit of the data. The Hill slope was defined as the slope of the relationship between Ca^{2+} and SERCA activity, obtained by using the portion of the SERCA activity curve that corresponds to 10-90% V_{max} .⁸ Additionally, we determined the relative amounts of passive Ca^{2+} leak across the SR membrane by comparing the ratio between V_{max} in the presence and absence ($V_{\text{max}(-)}$) of 1 μM Ca^{2+} -ionophore (ionophore ratio). Maximal SERCA activity (V_{max}) and $V_{\text{max}(-)}$ were both determined at pCa 6.13.

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References

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