Cardiovascular Research Center, Department of Surgery, Rhode Island Hospital and Alpert Medical School of Brown University, Providence, RI.

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From the Cardiovascular Research Center, Department of Surgery, Rhode Island Hospital and Alpert Medical School of Brown University, Providence, RI.

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Correspondence to Richard T. Clements, PhD, Cardiovascular Research Center, Department of Surgery, Rhode Island Hospital and Alpert Medical School, Brown University, 5230, 1 Hopkins St, Providence, RI 02903. E-mail rilements2@lifespan.org

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Rottlerin Increases Cardiac Contractile Performance and Coronary Perfusion Through BKCa++ Channel Activation After Cold Cardioplegic Arrest in Isolated Hearts

Richard T. Clements, PhD; Brenda Cordeiro, BA; Jun Feng, MD, PhD; Cesario Bianchi, MD, PhD; Frank W. Sellke, MD

Background—Cardioplegia and cardiopulmonary bypass (CP/CPB) subjects myocardium to complex injurious stimuli that can result in cardiomyocyte and vascular contractile abnormalities. Rottlerin, originally identified as a delta-protein kinase C inhibitor, has a number of known additional effects that may be beneficial in the setting of CP/CPB. We tested the hypothesis that rottlerin mitigates deleterious effects associated with CP/CPB.

Methods and Results—Langendorff-perfused isolated rat hearts were subjected to 2 hours intermittent cold (10°C) CP (St Thomas II) followed by 30 minutes normothermic reperfusion. CP was delivered every 30 minutes for 1 minute. Hearts were treated with rottlerin 1 μmol/L (CP+R) (n=7) or without rottlerin (CP) (n=9), and the BKCa++ channel inhibitor paxilline 100 nmol/L was supplied in the CP. Hearts constantly perfused with KHB served as controls (n=6). Baseline parameters of cardiac function were similar between groups. CP resulted in reduced cardiac function (left ventricular diastolic pressure, 39±3.8%; ±dP/dt, 32±4.4%, −41±5.1% decrease compared to baseline). Treatment with rottlerin 1 μmol/L significantly improved CP-induced cardiac function (left ventricular diastolic pressure, 20±5.9%; ±dP/dt, 5.2±4.5%, −11.6±4.7% decrease versus baseline; P<0.05 CP+R versus CP). Rottlerin also caused a significant increase in coronary flow postreperfusion (CP, 34±4.2% decrease from baseline; CP+R, 26±9.6% increase over baseline; P=0.01). Independent of vascular effects, CP significantly decreased isolated myocyte contraction, which was restored by rottlerin treatment. The BKCa++ channel inhibitor greatly reduced the majority of beneficial effects associated with rottlerin.

Conclusions—Rottlerin significantly improves cardiac performance after CP arrest through improved cardiomyocyte contraction and coronary perfusion. (Circulation. 2011;124[supp 1]:S55–S61.)

Key Words: cardioplegia ■ potassium channels ■ protein kinase C ■ rottlerin ■ Akt ■ ischemia

Cardiac surgery using cardioplegia (CP) and cardiopulmonary bypass subjects myocardium to hypothermic reversible ischemic injury that can impair cardiac function (aka, myocardial stunning). The main protective benefits of CP are mediated through myocardial hypothermia and diastolic arrest, which preserve myocardial energy reserves. The ischemic insults associated with CP arrest during surgery include myocyte hypoxia, acidosis, oxidant-dependent damage, metabolic and structural alterations, and reduced cardiac function.1–4 In addition to direct effects on cardiomyocytes, CP can result in marked coronary vascular complications, including impaired vasodilation, propensity for spasm, and overall decreased perfusion.5

Although contractile impairment in the majority of patients resolves quickly, 10% can develop a cardiac low-output syndrome attributable in part to depressed left ventricular (LV) or atrial contractile function. Consequently, low-output syndrome prolongs recovery times and significantly elevates risk of mortality.6 Furthermore, the need for enhanced cardioprotection is required for specific high-risk patient populations (eg, prolonged surgical times, low ejection fraction, older age).

Rottlerin has been reported as a protein kinase C-δ (PKCδ) inhibitor. PKCδ has been implicated in depressed cardiac function and cell death after ischemia-reperfusion injury as well as in promoting vascular smooth muscle contraction.7–9 However, rottlerin as a true inhibitor of PKCδ has been called into question and has generated considerable controversy.10,11 Other PKCδ-independent effects of rottlerin recently have been recognized. Rottlerin has been reported as a potent large
conundrable potassium channel (BK<sub>Ca<sup>++</sup></sub> opener). Opening of BK<sub>Ca<sup>++</sup></sub> channels is beneficial for postischemic alterations in vasomotor activity. In addition, other BK<sub>Ca<sup>++</sup></sub> channel openers are reported to limit ischemia-related mitochondrial Ca<sup>2+</sup> overload. Finally, rottlerin is capable of reducing oxygen radical formation. All these mechanisms of injury occur during CP arrest and reperfusion. Therefore, through a combination of targets, rottlerin may block many of the deleterious side effects associated with CP arrest that limit both vascular and cardiomyocyte function.

Methods

Isolated Langendorff-Perfused Model of CP Arrest

Male Sprague-Dawley rats (Charles River; Wilmington, MA) were anesthetized with 80 mg/kg ketamine IP and 5 mg/kg xylazine IP, and the heart was rapidly excised. The aorta was immediately cannulated and retrograde perfused in Langendorff mode with a water jacketed organ chamber and perfusion system (IH-SR; Harvard Apparatus, Inc; Holliston, MA). After cannulation, the heart was cleaned of excess tissue and vessels, the left atrium removed, and a balloon placed in the LV. LV end-diastolic pressure was set to >8 mm Hg at the beginning of the experiment. A temperature probe placed in the pulmonary artery monitored myocardial temperature. The hearts were perfused in constant pressure mode (~70 mm Hg) with a modified KHB (NaCl, 118 mmol/L; KCl, 4.7 mmol/L; CaCl<sub>2</sub>, 1.25 mmol/L; MgSO<sub>4</sub>, 1.66 mmol/L; NaHCO<sub>3</sub>, 24.88 mmol/L; KH<sub>2</sub>PO<sub>4</sub>, 1.18 mmol/L; Na-pyruvate, 2.0 mmol/L) for 30 minutes to stabilize and record baseline measurements. During baseline measurements, myocardial temperature was maintained at 37°C. Groups subjected to cold crystalloid CP solution were perfused with St Thomas II solution (NaCl, 110 mmol/L; KCl, 16 mmol/L; MgCl<sub>2</sub>, 1.5 mmol/L; CaCl<sub>2</sub>, 1.25 mmol/L; NaHCO<sub>3</sub>, 10 mmol/L). Myocardial cooling during CP was initiated at the onset of CP infusion through rapidly switching the Langendorff organ chamber and perfusate to a refrigerated circulator. Myocardial temperature was maintained at 10°C for the duration of CP. CP groups were perfused initially for 2 minutes followed by a 1-minute infusion at 30, 60, and 90 minutes-arrest, respectively. After 120 minutes, the organ chamber and perfusate were switched back to a heating circulator and the heart perfused at 70 mm Hg with modified KHB. Myocardial temperature was subsequently maintained at 37°C. Indices of ventricular function, perfusion pressure, myocardial temperature, and organ chamber temperature were measured continuously throughout the experiment using an LDS-Ponemah data acquisition system. At the conclusion of the experiment, the heart was removed from the perfusion apparatus, and a small midtransverse slice was removed and immediately placed in 10% formalin for confocal microscopy. A second section was taken for a heart wet/dry weight ratio. Samples were weighed directly after procurement and after desiccation for 24 hours at 50°C. The remainder of the tissue was immediately placed in liquid N<sub>2</sub>.

SDS-PAGE and Immunoblot

SDS-PAGE and immunoblot were performed using standard methodology as previously described. Antibodies for immunoblot were as follows: phosphospecific or total PKCδ, cardiac tropinin I (cTNI), Akt, 3-phosphoinositide-dependent kinase 1 (PDK1), phosphatase and tensin homolog (PTEN), and extracellular signal-regulated kinase (ERK) from Cell Signaling (Beverly, MA); phospholamban (PLN) from Millipore (Billerica, MA); and phospho- and total heat shock protein 27 (HSP27) and αβ-crystallin antibodies from Stressgen (Vancouver, BC).

Culture and Purification of Adult Rat Cardiomyocytes

Adult rat cardiomyocyte cultures were obtained from hearts according to a previously published protocol with modifications. Briefly, hearts were excised from anesthetized adult rats, the aorta cannulated, and hearts perfused with 0.3% collagenase solution in perfusion buffer consisting of MEM (Joklik modification) supplemented with creatine, BDM, taurine, and insulin for 45 minutes. After perfusion, ventricles were removed and minced in the Ca<sup>2+</sup>-free collagenase solution for 3 to 5 minutes. Chunks were then incubated in 10 mL of perfusion buffer supplemented with BSA and 0.3 mmol/L CaCl<sub>2</sub>. Cells were washed in collagenase-free perfusion buffer 3 times with centrifugation. Myocytes were resuspended in DMEM culture medium supplemented with creatine, carnitine, taurine, penicillin/streptomycin, and insulin. Myocytes were plated at a density of 2×10<sup>6</sup> cells/cm<sup>2</sup> on 10 μg/mL laminin-coated dishes for length measurements the following day.

In Vitro CP and Myocyte Length Recordings

Cultured rat myocytes were switched to crystalloid CP solution bubbled with 5% CO<sub>2</sub>/95% N<sub>2</sub> under anoxic conditions. Cells were then placed in a hypoxia chamber evacuated with 5% CO<sub>2</sub>/95% N<sub>2</sub> for the indicated times and retained at 4°C. For simulated reperfusion, CP-treated cells were removed from the hypoxic chamber and returned to the cell culture incubator, and CP solution was replaced with a modified KHB containing NaCl, 118 mmol/L; KCl, 4.7 mmol/L; CaCl<sub>2</sub>, 1.25 mmol/L; MgSO<sub>4</sub>, 1.2 mmol/L; KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol/L; NaHCO<sub>3</sub>, 25 mmol/L; HEPES, 10 mmol/L; and glucose, 10 mmol/L. Control cells were maintained in a standard cell culture incubator for the duration of CP and switched to 37°C KHB at the time of simulated reperfusion. For length measurement, cells were moved to a heated stage with temperature-controlled perfusion and field stimulation capable of accepting 35-mm culture dishes (Harvard Apparatus). The cells were imaged with a CCD camera-equipped inverted microscope attached to a raster line video edge detector (Living Systems Instrumentation; Burlington, VT). Voltage signals were captured with a data acquisition unit attached to a computer running Ponemah physiology platform software. The recorded length data can be visualized in real time at 60 Hz by computer monitor and stored for subsequent analysis of percent length change and positive and negative first derivatives of LV pressure (±dP/dt). The video edge detector and data capture software were calibrated using voltage signals from a slide micrometer before each experiment. Only rod-shaped viable cells that responded appropriately to field stimulation were used.

Statistical Analysis

All statistical analyses were performed with Sigma Stat software (Systat Software Inc; Chicago, IL). For analysis of CP and CP+rottlerin (CP+R) functional time courses, 2-way repeated-measures ANOVA with Student Newman-Keuls post hoc analysis was performed. A 2-way ANOVA with Student Newman-Keuls was performed for isolated myocyte experiments. All other statistical tests were 1-way ANOVA with Student Newman-Keuls. P<0.05 determined significance.

Results

Rottlerin Alleviates CP-Induced Myocardial Stunning in the Isolated Heart

Cold crystalloid CP for 2 hours in the isolated rat hearts caused depressed cardiac contractile function on reperfusion. During CP, myocardial temperature was maintained at ~10°C, followed by rewarthing to 37°C during 30 minutes of reperfusion as described previously. A representative experiment is presented in online-only Data Supplement Figure 1. On reperfusion, there were significant reductions in systolic pressure, developed pressure, and ±dP/dt compared with baseline (Figure 1A through 1D). Inclusion of rottlerin 1 μmol/L in the CP solution significantly improved indices of cardiac function, including developed pressure (Figure 1A), ±dP/dt (Figure 1B and 1C), and tau (Figure 1D).
There were no significant changes in heart rate between groups (Figure 1E).

**Rottlerin Improves CP-Induced Reductions in Coronary Perfusion**

CP arrest and 30 minutes reperfusion reduced coronary flow compared with baseline. Hearts treated with rottlerin 1 μmol/L in the CP solution showed significant improvements over CP alone (Figure 2A and 2B). At 30 minutes of reperfusion, the CP+R group showed a significant increase in flow over baseline, whereas CP and sham hearts showed overall reductions in flow (Figure 2B). There were no significant increases in wet/dry tissue weight ratios between groups, indicating no deleterious increases in tissue edema (online-only Data Supplement Figure 2).

**Rottlerin Blocks CP-Impaired Myocyte Contractility In Vitro**

To explore whether rottlerin alleviates CP-induced depressed contractile function solely through vascular effects, isolated rat cardiac myocytes were subjected to in vitro CP and simulated reperfusion with reoxygenation and rewarming. In vitro CP resulted in depressed myocyte contraction as determined by decreased myocyte length.
shortening (Figure 3A and 3B). Inclusion of rottlerin 1 μmol/L completely rescued CP-induced changes in myocyte length shortening (Figure 3A and 4B), indicating direct effects on cardiomyocytes.

Cardioprotective Effects of Rottlerin Are Independent of PKCδ
PKCδ phosphorylation is known to correlate with increased PKCδ activity. However, CP did not increase the phosphorylation of PKCδ on Y311, T505, or S643. There was an insignificant trend for CP-induced phosphorylation of PKCδ-T505, but treatment with rottlerin 1 μmol/L did not attenuate this trend or decrease basal phosphorylation on any of the residues examined (Figure 4A and 4B). Phosphorylation of ERK and p38-MAPK/HSP27 have been implicated as downstream of PKCδ activation. HSP27 is a downstream target of p38-MAPK that also may regulate contractile deficits associated with CP-induced stunning. Although CP induces phosphorylation of ERK and HSP27, these effects were independent of rottlerin (Figure 4C and 4D).

BKCa++ Channels Mediate Rottlerin-Induced Improvements in Post-CP Cardiac and Vascular Function
To determine whether rottlerin mediates its effects through the opening of BKCa++ channels, hearts were treated with rottlerin 1 μmol/L and the BKCa++ channel blocker paxilline 100 nmol/L and 1 μmol/L, both supplied in the CP condition. Paxilline blocked the majority of beneficial effects of rottlerin at 100 nmol/L and all the measured beneficial effects at 1 μmol/L (Figure 5). However, treatment with rottlerin and paxilline 1 μmol/L caused significant increases in LV end-diastolic pressure and tau compared with CP treatment alone. Full-time courses of the paxilline-treated groups are presented in online-only Data Supplement Figure 3.

Rottlerin Enhances CP-Induced Akt Phosphorylation Through BKCa++ Channels
CP caused significant phosphorylation increases in members of the Akt signaling cascade, including Akt ser473, Akt thr308, ser241 PDK1, and ser380 PTEN (Figure 6). Compared with CP alone, rottlerin significantly increased the phosphorylation of Akt on thr308. All targets of the Akt pathway measured were significantly reduced in the rottlerin groups cotreated with paxilline 100 nmol/L (Figure 6), indicating that this pathway is controlled by BKCa++ channel modulation.

Rottlerin Does Not Reduce Protein or Lipid Oxidation
To determine whether rottlerin mediates its effects through antioxidant mechanisms, tissue protein and lipid oxidation were indirectly measured by carbonyl and malondialdehyde content, respectively. Neither CP nor addition of rottlerin significantly altered protein (online-only Data Supplement Figure 4A) or lipid oxidation (online-only Data Supplement Figure 4B) compared with sham treatment.
Rottlerin Does Not Alter PLN or cTnI Phosphorylation

Phosphorylation of PLN and cTnI are implicated in regulation of myocardial contractility. Neither CP nor CP/R 1 \( \mu \)mol/L changed basal levels of cTnI or PLN phosphorylation (online-only Data Supplement Figure 5).

Discussion

The principle findings of the current study indicate that rottlerin improves functional recovery of isolated hearts after cold CP arrest. As an additive to CP, rottlerin (1) increased isolated heart contractile performance, (2) enhanced significantly myocardial perfusion, and (3) directly increased contractile performance of cardiac myocytes independent of vascular effects. All of these beneficial effects appeared to be independent of PKC\(\delta\) activation as measured by phosphorylation status and screening of potential downstream targets. In contrast, the beneficial effects of rottlerin were reduced by paxilline, implicating an important role for BKCa channels.

CP arrest can result in multiple complications after surgery, including enhanced coronary artery tone and decreased myocardial perfusion as well as perfusion-independent effects that cause myocardial stunning and contractile deficits. Rottlerin may mediate improvements in cardiomyocyte and smooth muscle function through known mechanisms of action that are independent of PKC\(\delta\).

First, rottlerin has been identified as an extremely potent BKCa opener.\(^{12}\) Activation of BKCa channels results in coronary smooth muscle vasodilation.\(^{13,23}\) Further, activation of BKCa channels with NS1619 (another BK channel activator) demonstrated improved vasodilation in vessels contracted with high K\(^\pm\) CP.\(^{13}\) Inhibition of the BKCa+ channels by guest on April 18, 2017 http://circ.ahajournals.org/ Downloaded from...
channels with paxilline greatly reduced the vasodilatory effect of rottlerin, indicating that rottlerin also works through smooth muscle BK_{Ca^{++}} channels to improve coronary flow after CP. Second, rottlerin-induced activation of BK_{Ca^{++}} channels may directly alleviate stunning of cardiac myocytes. Classical mechanisms of myocardial stunning associated with CP and cardiopulmonary bypass include the oxidant radical and Ca^{2+} overload hypothesis.\textsuperscript{2,4,6,24} The nonexclusive views propose (1) oxidant-dependent damage/oxidant-dependent activation of signaling, which negatively modulate the contractile apparatus, and (2) alterations in Ca^{2+} homeostasis, which promote contractile abnormalities and metabolic alterations through mitochondrial damage. BK_{Ca^{++}} channels reside in the cardiomyocyte inner mitochondrial membrane.\textsuperscript{25} BK_{Ca^{++}} channel activation is proposed to increase mitochondrial Ca^{2+} accumulation, which, in turn, electrochemically limits mitochondrial Ca^{2+} influx and reduces mitochondrial depolarization and permeability transition pore opening.\textsuperscript{14,15}

Known cardioprotective effects associated with the BK_{Ca^{++}} channel openers NS1619, NS11021, and DiCl-DHAA after ischemia-reperfusion injury include reductions in mitochondrial Ca^{2+} overload, mitochondrial membrane depolarization, increased cell viability, and improved function in whole hearts.\textsuperscript{15,25,26} Indeed, adaptation to hypoxia alone can partially activate the mitochondrial BK_{Ca^{++}} channels, which is recognized as a protective response.\textsuperscript{27,28}

In addition, we found that rottlerin treatment greatly enhanced the CP-induced phosphorylation of Akt on the required activation residue thr308. Akt activation is reported to be an upstream mediator of mitochondrial K\textsuperscript{+} channel function as well as a modulator of the mitochondrial depolarization and permeability transition pore.\textsuperscript{29,30} However, the present data indicate that Akt functions downstream of rottlerin-dependent activation of BK_{Ca^{++}} channels because BK_{Ca^{++}} channel inhibition with paxilline blocked the CP and CP+R-induced increases in Akt pathway activation (Figure 6). Although activation of Akt signaling is considered beneficial and prosurvival after ischemia-reperfusion injury, it is unclear what specific role (if any) Akt may play in modulating the acute increases in myocardial function after CP+R treatment. Further studies will need to address whether this aspect of improved function associated with rottlerin is necessary.

Rottlerin has demonstrated antioxidant properties, although it is unclear whether these effects are due to BK_{Ca^{++}} opening or other additional mechanisms.\textsuperscript{10,31} However, we did not find any evidence that rottlerin directly influences oxidant-dependent damage as measured by total lysate protein and lipid oxidation. Specific investigation of these effects may be required in isolated mitochondria and with more sensitive methods. Finally, it is highly likely that the dose of rottlerin used in the present studies did not inhibit PKC\textepsilon. Previous studies demonstrated that PKC\textepsilon inhibitory doses are significantly >1 \mu mol/L. Numerous reports indicated that rottlerin may indirectly alter PKC\textepsilon activation but at concentrations (ie, >10 \mu mol/L) that lead to mitochondrial uncoupling and global reductions in ATP and subsequent kinase activity.\textsuperscript{10,11,32} However, the dose of rottlerin used in the current study is considerably less than any dose that was previously shown to alter cellular ATP levels and lead to activation of AMPK.\textsuperscript{32} In addition, we found no activation of AMPK with or without rottlerin in the CP-treated groups, indicating preservation of mitochondrial energy reserves (data not shown). We also found no phosphorylation of PKC\textepsilon or potential downstream targets. Conversely, rottlerin 0.5 \mu mol/L significantly increases BK_{Ca^{++}} channel opening in cell and cell-free systems.\textsuperscript{12}

Limitations

Although the present model sought to faithfully reproduce injury associated with CP arrest in patients, a number of important shortcomings exist. First, we did not address injury associated with extracorporeal circulation and reperfusion injury associated with blood components. Second, the most common form of CP currently in use is cold blood crystalloid CP; thus, the effect of blood as a CP additive on our results is unclear. Future studies will need to address the potential benefit of rottlerin with a model that incorporates CP arrest and reperfusion after cardiopulmonary bypass.

Conclusions

The present study demonstrates that rottlerin, as an additive to cold crystalloid CP, improves CP-induced myocardial stun-
ning and vasomotor regulation likely through direct activation of BK_{Ca++} channels and not PKCδ-dependent effects. To our knowledge, this is the first use of rottlerin and a BK_{Ca++} activator in a whole-heart model of CP arrest, and we demonstrate improved cardiomyocyte and vascular function after reperfusion. These results indicate that rottlerin as a CP additive with no requisite before and after treatment may provide significant therapeutic benefit for cardiac surgery patients.

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Disclosures
None.

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Supplemental Material:

Supplemental Methods

**Protein and Lipid Oxidation:** Protein carbonyl formation was measured using the Oxyblot system (Millipore) according to the manufacturer’s instructions and as described previously (1). Malondialdehyde (MDA) content indicative of lipid peroxidation was measured using a Bioxytech MDA-586 kit (OxisResearch, Burlingame, Ca) according to the manufacturer’s instructions and as described previously (1).

Supplemental Figure Legends

**Figure Legends:**

Supplemental Figure 1 – Cold crystalloid cardioplegia reduces cardiac performance. A) Representative experiment of cardioplegia/reperfusion in isolated rat hearts. a. – baseline measurement b. – induction of cardioplegia (2 min delivery) , c) Cardioplegic arrest 30 min (1 min CP delivery) d 60 min Cardioplegic arrest (1 min CP) e. 90 min CP (1 min delivery) f. Start reperfusion. g-h: 5, 10, 20 ,30 min reperfusion. B) Tracings from individual hearts subjected to CP and reperfusion with or without 1 uM Rottlerin.

Supplemental Figure 2. Rottlerin does not alter wet/dry tissue weight. None of the treatments significantly effected wet/dry tissue weights indicating no gross increases in tissue edema. Minimum n=6, except CP + Rott + Paxilline 1 uM , n=4. One Way Anova,.

Supplemental Figure 3. The BKCa++ channel inhibitor paxilline blocks beneficial effects of rottlerin on post-CP cardiac function. Experimental conditions similar to figures 1 and 2. Cotreatment with rottlerin (1 uM) and Paxilline (100 nM or 1 uM) supplied as an additive to cardioplegia. A) Developed Pressure, B) +dP/dt, C) –dP/dt, D) Tau, and E) LVEDP F) Heart rate and G) Coronary Flow. X-axis time : Baseline – pre CP
function, CP – Cardioplegia, 5 - 30 – min reperfusion. n = minimum of 6 per group, except Rottlerin + 1 uM Pax, n=4. One Way ANOVA, Student Newman-Keuls.

**Supplemental Figure 4**– **Rottlerin does not significantly alter protein or lipid oxidation.** A Representative oxyblot and graph of normalized fold increase over sham in protein carbonyl content measured by oxyblot. B) Graph of lipid oxidation measured by tissue MDA content. Graphs minimum n=6 / group. One Way ANOVA.

**Supplemental Figure 5**– **Rottlerin does not significantly alter phospholamban or troponin I phosphorylation.** Graph displays normalized fold increase in phosphorylation over sham. Minimum n=6 / group. One Way ANOVA.

**Reference List**

Supplemental Figure 1

A

LVP (mmHg)

\[ \text{dP}/\text{dt} \] (mmHg)

myocardial temp (deg C)

baseline  cardioplegia 2 hours  reperfusion 30 min

B

CP

CP + Rott

Perfusion pressure

LVP

dP/dt
Supplemental Figure 2

![Graph showing Wet: Dry Weight Ratio for different conditions: Sham, CP, CP + Rott, CP + Rott + 100nm Pax, CP + Rott + 1uM Pax. The graph indicates varying levels of wet to dry weight ratio for each condition.](image-url)
Supplemental figure 4

A

Normalized Protein Oxidation

B

µM MDA
Supplemental figure 5