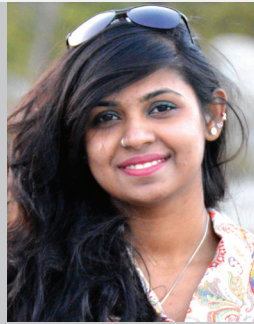


# REDUCING RISK OF CARDIOVASCULAR DISEASE

## Student Author

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## Abstract

Calcium plays an important role in regulating the contraction of smooth muscles. Membrane potential of the smooth muscles regulates the smooth muscle contractility through variation in  $\text{Ca}^{2+}$  influx with the assistance of the voltage-operated  $\text{Ca}^{2+}$  channel. Using the porcine pulmonary arteries (PPA) as the model system, the effect of the antioxidant quercetin on the regulation of pulmonary arteries was investigated in the presence and absence of calcium. It was hypothesized that quercetin reduces the contractile force in PPA by limiting  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum (SR) without imposing any effect on the voltage-operated channel (VOC). However, the biochemical pathway of quercetin is still unknown. In this study, it is shown that PPA contracts both in the presence and absence of  $\text{Ca}^{2+}$ . Further investigations are required to study the exact effects of KCl in 0  $\text{Ca}^{2+}$  to completely break down the biochemical pathway of quercetin and reassure its effect on the SR. Since quercetin demonstrates reduction in contractile force, it has high potential to provide a solution for the side effects of hypertension, which is a complex heterogeneous disorder that can coexist with other cardiovascular abnormalities.

## Mentor

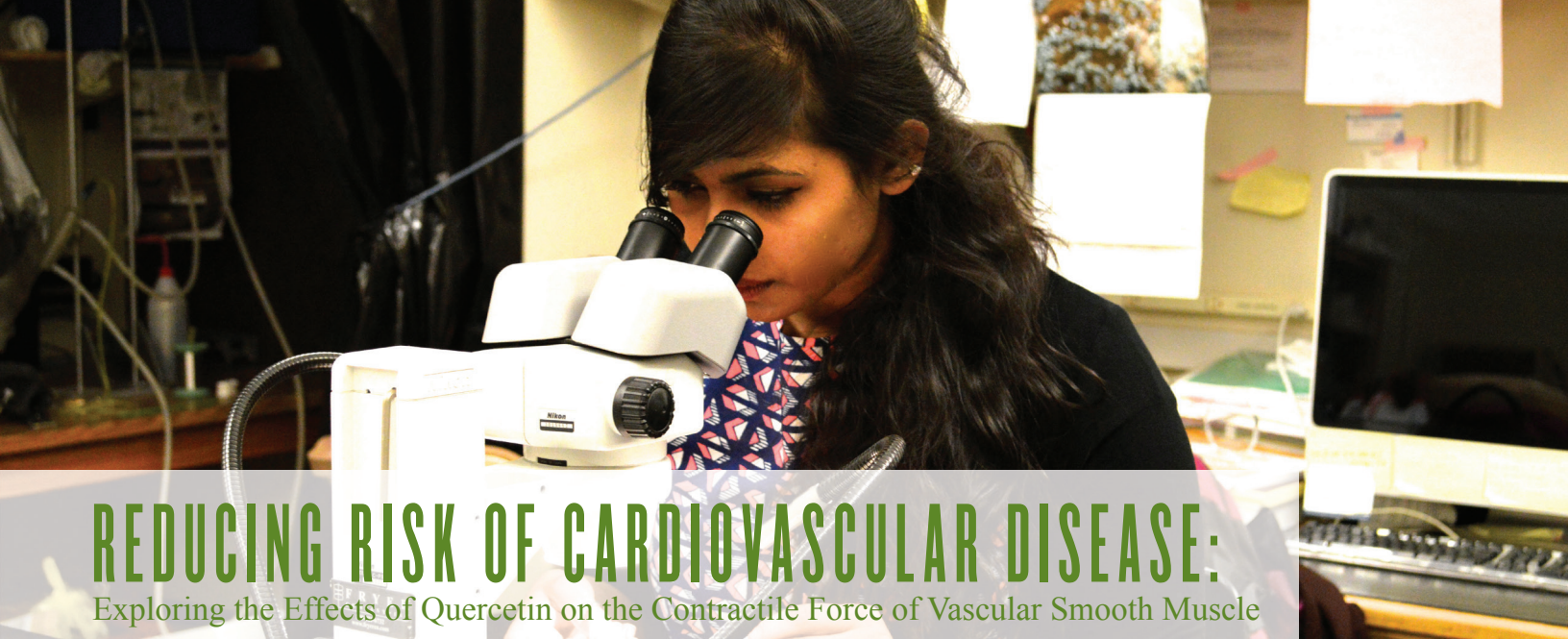
**Nancy Pelaez** received a BS in biology summa cum laude from Newcomb College of Tulane University in New Orleans, a K–12 California single-subject teaching credential in both life science and physical science from Mills College in Oakland, California, and, with support from a Howard Hughes Medical Institute Fellowship, a PhD in physiology and biophysics with a research focus on vascular muscle physiology from Indiana University School of Medicine. Pelaez, who founded the Purdue International Biology Education Research Group (PIBERG), is currently a member of the Fulbright Roster of Scholars and consults on biology education research projects overseas.



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## Keywords

depolarization, porcine pulmonary artery, calcium, quercetin, Nernst Equation, cardiovascular, voltage-operated channel, contraction, influx, extracellular



# REDUCING RISK OF CARDIOVASCULAR DISEASE:

Exploring the Effects of Quercetin on the Contractile Force of Vascular Smooth Muscle

Puja Banerjee, Engineering and Biochemistry

## INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of death in men and women, killing around 787,000 people in the United States alone in 2011 (Heart Foundation, n.d.). CVD claims more lives than all forms of cancer combined, which totaled around 571,950 deaths in 2011. These numbers indicate that CVD is becoming an epidemic (Tanoue, n.d.). Although some solutions to CVD already exist, there is room for investigation of how to prevent this disease before it happens. This research focuses on finding a prospect solution for reducing the risk of CVD by investigating the effect of quercetin on the contraction of porcine pulmonary arteries (PPA).

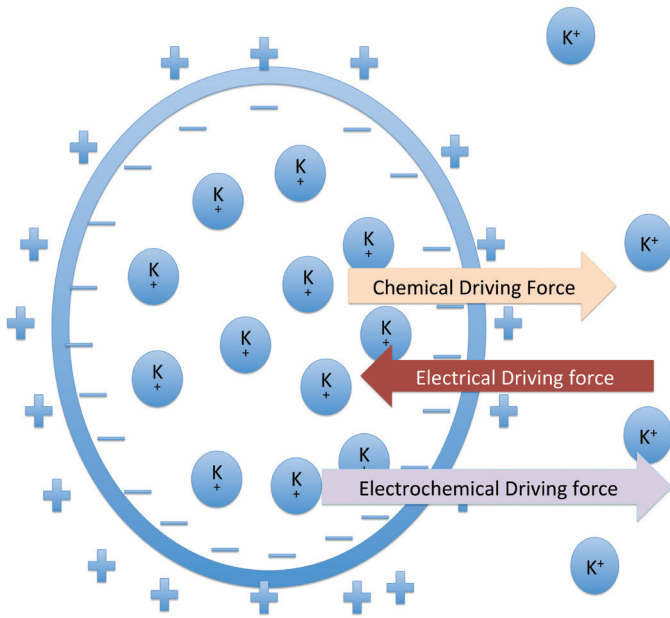
Quercetin is a polyphenolic flavonoid that has revealed its potential as a health-promoting, disease-preventing supplement. It is readily available in apples, onions, berries, and red wine. Epidemiological studies have found an inverse relationship between dietary quercetin intake and CVD; increased intake of dietary food rich in quercetin has been linked with decreased heart disease and stroke incidences. It also is a natural antihistamine, reduces platelet aggression, facilitates low blood pressure, increases immunity, and modulates inflammation. Because of all of these effects, quercetin has the ability to reduce factors that contribute to CVD, such as hypertension, diabetes, and hyperlipidemia.

In order to determine the mechanism by which quercetin exerts cardioprotective effects, other studies have used *in vitro*, *in vivo*, and clinical research. It has been reported that quercetin inhibits the  $\text{Ca}^{2+}$  dependent activity and also the uptake of  $\text{Ca}^{2+}$  by the sarcoplasmic reticulum (SR)

(Shoshan, 1980). In 2004, British researchers from the University of Kiel demonstrated that quercetin reduced platelet aggression (Hubbard, Wolfram, Lovegrove, & Gibbins, 2004), implying that quercetin was linked with reduced risk of blood clotting. Greek cardiologists concluded from their experiments that red grape polyphenol extract that was rich in quercetin caused an increase in flow-mediated dilation of major arteries, which facilitates low blood pressure (Lekakis et al., 2005). Moreover, a study undertaken at the University of Utah also demonstrated reduction of blood pressure due to quercetin supplements (Edwards et al., 2007). Taken together, these results suggest that quercetin supplementation might be a viable solution to CVD. Hence, this research investigates the effect of quercetin on the force of contraction of PPA with the ambition of finding a way to prevent CVD.

## Physiological Roles of $\text{K}^+$ Channels in Vascular Smooth Muscle

Pulmonary arterial muscle cells respond through changes in their membrane potential. The membrane potential rises due to the presence of ions of different concentrations between the exterior and interior of the cell, which are separated by lipid bilayers. There are ion channels and ion pumps across the membrane that assist the movement of the ions to maintain the concentration gradient. In living smooth muscle cells, the inside of the cell has a higher concentration of the potassium ion ( $\text{K}^+$ ) than the outside. Due to differences in the concentration gradient across the cell membrane,  $\text{K}^+$  diffuses out of the cell down its concentration gradient with the assistance of ion channels as shown in Figure 1. This develops an electrical potential difference across the membrane, making the interior of the cell more negatively charged.



**Figure 1.** K<sup>+</sup> moving out of the cell due to difference in concentration gradient.

In response to the electrical potential difference across the cell membrane, a negative charge attracts K<sup>+</sup> ions toward the inside of the cell even though the K<sup>+</sup> concentration is higher inside the cell until an equilibrium potential is achieved for K<sup>+</sup>. This equilibrium potential, also known as the Nernst equilibrium voltage (V<sub>n</sub>), counterbalances diffusion due to the concentration difference of K<sup>+</sup> across the membrane. The equilibrium potential voltage for K<sup>+</sup> ions can be calculated using the Nernst equation, which is  $V_n = RT/zF (c_0/c_i)$  (Klabunde, 2012).

The ion transport facilitated by ion channels is passive, and the ion flux depends on V<sub>n</sub> and the permeability of the channel for particular ions. The flow of K<sup>+</sup> ions through the membrane channel is driven by the voltage gradient of all ions across the membrane and the concentration gradient of K<sup>+</sup> ions. Therefore, V<sub>n</sub> of smooth muscle regulates muscle contraction through alteration in Ca<sup>2+</sup> influx via voltage-operated Ca<sup>2+</sup> channels. Depolarization begins when the electrical potential across the cell membrane shifts to a less negative value. The membrane can be depolarized by changing the K<sup>+</sup> ion concentration outside the cell membrane (Ny, 1996).

### KCl-induced Contraction of Vascular Smooth Muscle

In this study, the smooth muscle tissues were placed in a standard Krebs-Henseleit solution bath with a concentration of 5.9 mM KCl. This resulted in a V<sub>n</sub> for K<sup>+</sup> of -71.3 mV, which was found using the Nernst equation as illustrated in Table 1. The cell membrane was submaximally contracted by the addition of 20 mM KCl that gives a

V<sub>n</sub> of -34 mV across the membrane, resulting in partial membrane depolarization. When the concentration of the extracellular K<sup>+</sup> was increased to 120 mM KCl, the V<sub>n</sub> of 0 mV was generated to maximally depolarize the membrane.

C <sub>i</sub>	C <sub>o</sub>	V <sub>n</sub> using Nernst equation	Result
100 mM	5.9 mM	$V_n = 58 \log(5.9/100)$	-71.3 mV
100 mM	15.9 mM	$V_n = 58 \log(15.9/100)$	-46 mV
100 mM	25.9 mM	$V_n = 58 \log(25.9/100)$	-34 mV
100 mM	125.9 mM	$V_n = 58 \log(125.9/100)$	5.8 mV

**Table 1.** The Nernst equation has been used to calculate the equilibrium potential with respect to change in extracellular K<sup>+</sup> concentration.

Therefore, depolarization of the membrane is achieved by increasing the extracellular K<sup>+</sup> concentration over the threshold level; not reaching threshold level would mean that voltage-operated channels (VOC) remain closed and induce relaxation to the muscles. Reaching the threshold K<sup>+</sup> level opens the L-type voltage gated Ca<sup>2+</sup> channels, resulting in an influx of Ca<sup>2+</sup> down the concentration gradient.

However, in the permanent presence of high extracellular K<sup>+</sup> concentration, most of the K<sup>+</sup> channels will not affect V<sub>n</sub> before the extracellular K<sup>+</sup> concentration is decreased and the VOC closes. When extracellular K<sup>+</sup> gradually decreases, these K<sup>+</sup> channels are effective again as the V<sub>n</sub> changes closer to the resulting potential and hyperpolarizes. The calculation of V<sub>n</sub> and a graphical representation of V<sub>n</sub> with the extracellular K<sup>+</sup> are presented in Table 1.

### Calcium-sensitive K<sup>+</sup> Channels

Calcium plays a major role in contraction of vascular smooth muscle (VSM). The increase of intracellular Ca<sup>2+</sup> can occur either from the influx of Ca<sup>2+</sup> ions through Ca<sup>2+</sup> channels or by the release of Ca<sup>2+</sup> from the sarcoplasmic reticulum. The Ca<sup>2+</sup> influx is achieved by electrical depolarization of the VSM cell membrane, which increases the intracellular concentration of Ca<sup>2+</sup> and contracts the VSM.

Moreover, a number of chemical stimuli such as norepinephrine, angiotensin II, vasopressin, endothelin-1, and thromboxane A2 can cause contraction by binding to specific receptors on the VSM (Ny, 1996). This mechanism involves different signal transduction pathways, all of which converge to increase intracellular calcium, as shown in Figure 2. The free intracellular calcium binds to calmodulin, which is a calcium-binding protein. Calcium-calmodulin activates myosin light chain kinase (MLCK), which



phosphorylates myosin light chains (MLC) with the assistance of ATP. The addition of the phosphate group results in the formation of a cross-bridge between myosin heads and actin filaments, leading to contraction. The relaxation of VSM is a result of a reduction in phosphorylation of MLC. This normally occurs due to a reduction of calcium entering into the cell from extracellular influx or the calcium release from the sarcoplasmic reticulum (Ny, 1996).

In this experiment, the effect of KCl-induced contraction of pulmonary arteries in a  $Ca^{2+}$ -free Krebs-Henseleit buffer containing EGTA was investigated. However, it is still not understood how the contraction occurs, since the  $Ca^{2+}$  ions over the plasma membrane were considerably lower than the cytosolic  $Ca^{2+}$  concentration. This result thus needs further investigation.

Additionally, VSM contraction also can take place without signal transduction pathways converging to increase intracellular  $Ca^{2+}$ . It is known that isolated vascular smooth muscle contracts in response to  $H_2O_2$ , independent of the  $Ca^{2+}$  signals.  $H_2O_2$ -induced contraction

appears to be a general response of vascular muscle (Pelaez, Braun, Paul, Meiss, & Packer, 2000). It is conserved across evolution, but the steps in the signal transduction pathway remain unknown.

## MATERIALS AND METHODS

Considering the chemistry behind the regulation of PPA and the capabilities of quercetin, we hypothesize that quercetin reduces contractile force by blocking  $Ca^{2+}$  release from the sarcoplasmic reticulum calcium stores in porcine pulmonary smooth arterial muscle, but has no effect on contractile force due to  $Ca^{2+}$  influx in porcine pulmonary smooth arteries. This prediction was verified by measuring the isometric force of the contracting arteries in isometric muscle baths of different compositions.

In our experiment, hogs were sacrificed and their fresh lungs and hearts were collected. Once the lungs were carefully separated from the gastrointestinal tract and the heart, they were completely submerged in an ice-cold Krebs-Henseleit buffer, which was previously bubbled

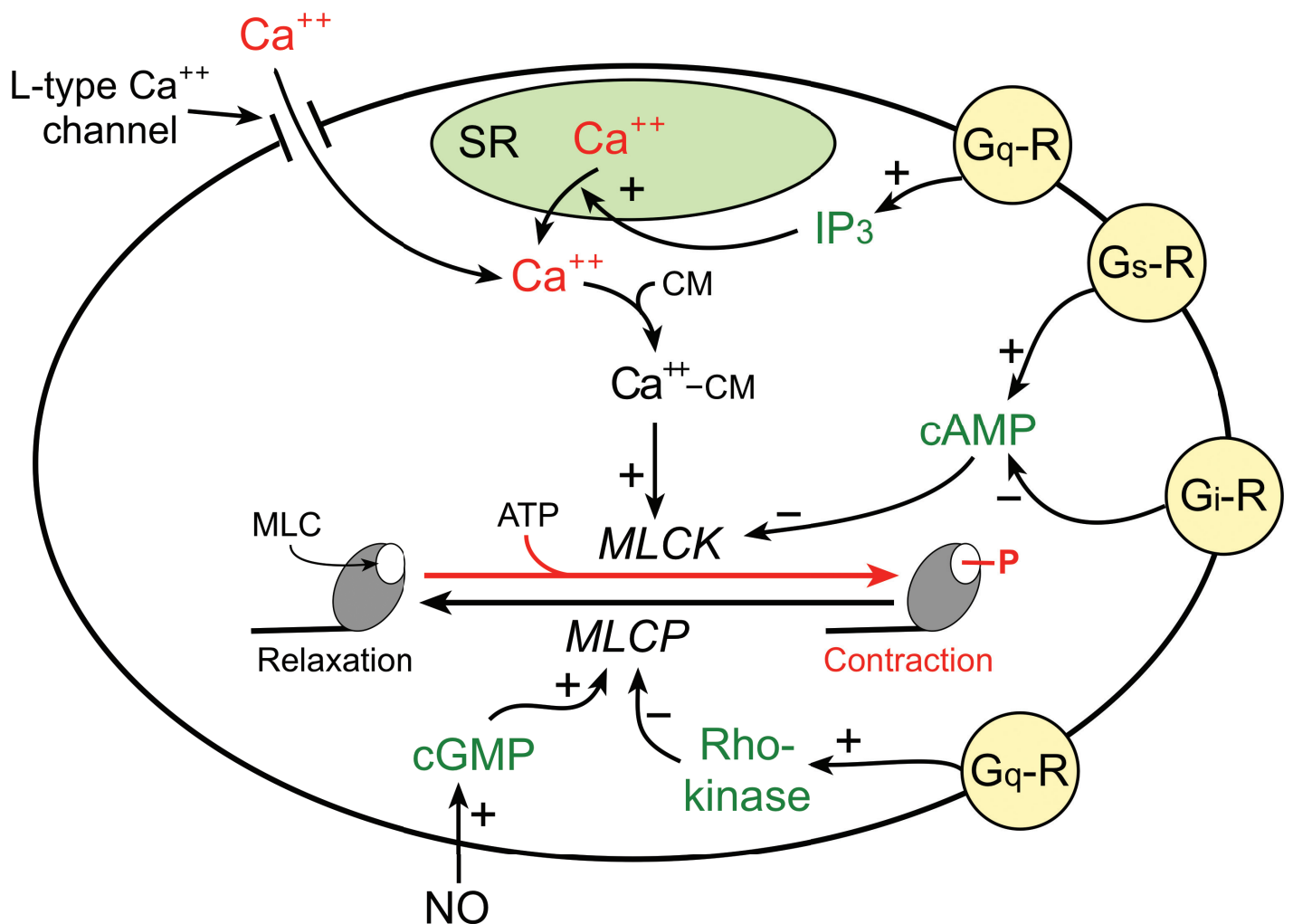
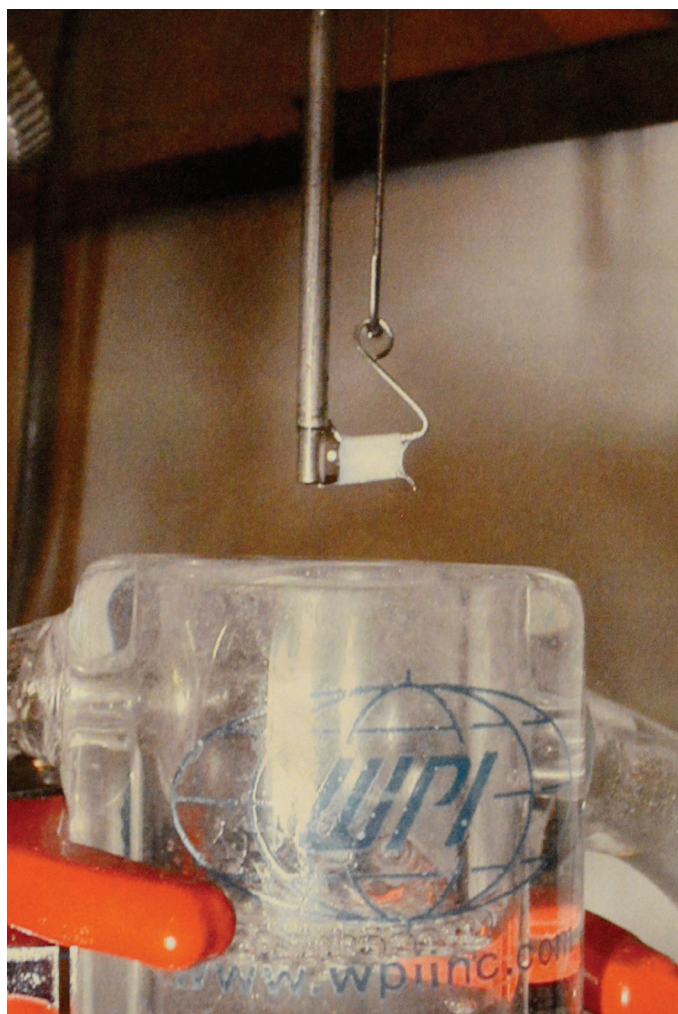


Figure 2. The pathway of vascular smooth muscle contraction.

with 95% O<sub>2</sub> and 5% CO<sub>2</sub> to make a solution for tissue preservation. The submerged lungs were transported to the lab where they were dissected to collect large vessels such as pulmonary arteries. These vessels were used to carry out our experiments to determine the contraction of pulmonary vascular muscles. The resistance vessel included the third- and fourth-generation pulmonary arteries. The tissues were submerged in an ice-cold Krebs-Henseleit buffer, and all the visible adhering connective tissue and parenchyma were cleaned under a dissecting microscope.

The arteries were cut into rings of diameter 3 mm. After dissection, the tissues that were not used immediately were stored in the refrigerator under MOPS solution. The equipment used to investigate tension of responding muscles was warmed for two hours before the start of the experiments. The arteries were placed in the tissue bath containing 10 mL of modified Krebs-Henseleit buffer solution bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Muscle rings of diameter 3 mm and length 5 mm were gently threaded on to a horizontally oriented fixed-position surgical steel



**Figure 3.** Muscle rings anchored to the wires.



**Figure 4.** Muscle rings submerged in Krebs-Henseleit solution.

wire. Once it was anchored, a second wire of the same dimensions that was connected to a force transducer was introduced into the lumen above the stationary wire as shown in Figure 3. The isometric force from contracting arteries submerged in the Krebs-Henseleit solution, as shown in Figure 4, were recorded digitally as a function of time on a PowerLab (ADInstruments) with the Lab Chart 7.0 software using a Harvard Force Transducer.

Stretching the arterial ring along its transverse axis increased the force measured by the transducer because these stretches led to changes in circumferential length. Each vascular ring was stretched similarly to produce the mean optimal resting tension (RPo) of 1.2 g for maximum active tension development (Po). Vascular rings were briefly contracted with the addition of 120 mM of KCl to ensure the tissues were alive. After five minutes they were washed with fresh Krebs-Henseleit buffer.

The vascular rings were allowed to relax to the baseline tension after the washout. They then warmed up and equilibrated for an hour, being stretched as needed to maintain the RPo at 1.2 g for each vessel. At the end of one hour, the vessels were supermaximally contracted with 120 mM of KCl and incubated for 40 minutes in order to reach a plateau. Once the plateau was reached, half of the vessels were washed with 10 mL Krebs-Henseleit buffer and the other half were washed with 10 mL zero-calcium Krebs-Henseleit buffer. They were then left for the next 40 minutes to let the tissues completely relax. 40 mM of KCl was added to submaximally contract all of the tissue. A solution of 60 mM quercetin was made by adding 80  $\mu$ L of 0.5 M quercetin to 10 mL of ethanol. This was added to the Krebs-Henseleit bath after 20 minutes.

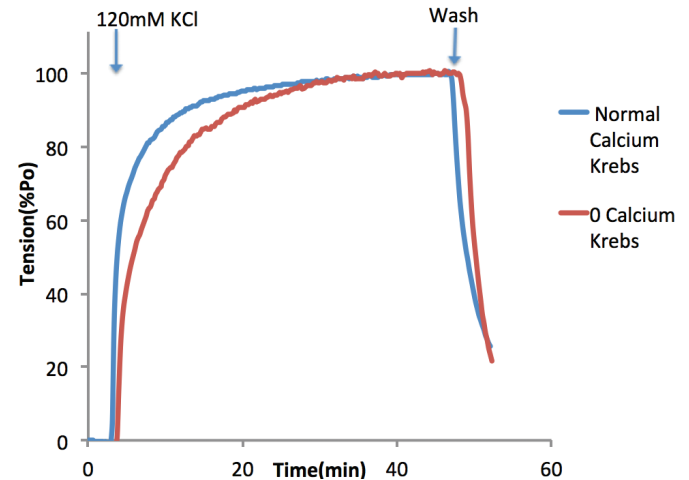
Some of the arteries served as the control (without quercetin) and some were the experiment (with quercetin). Half of those with calcium and half of those without calcium were treated with quercetin. Simultaneously, 80  $\mu$ L of ethanol was added to the other half with calcium and without calcium. This allowed us to find out if the effect of quercetin was due to ethanol, because the solution of quercetin had ethanol in it as the solvent. The effect of quercetin was observed for the next 20 minutes and then 80 mM more of KCl was added (80 + 40 = 120 mM) to investigate if the antioxidant quercetin altered the contractile function of the tissue. After 40 minutes, all the channels were washed with fresh Krebs-Henseleit buffer. After waiting about 30 minutes, 120 mM KCl was added to check for contraction. If the muscles contracted, then the arteries were still alive.

## RESULTS AND DISCUSSION

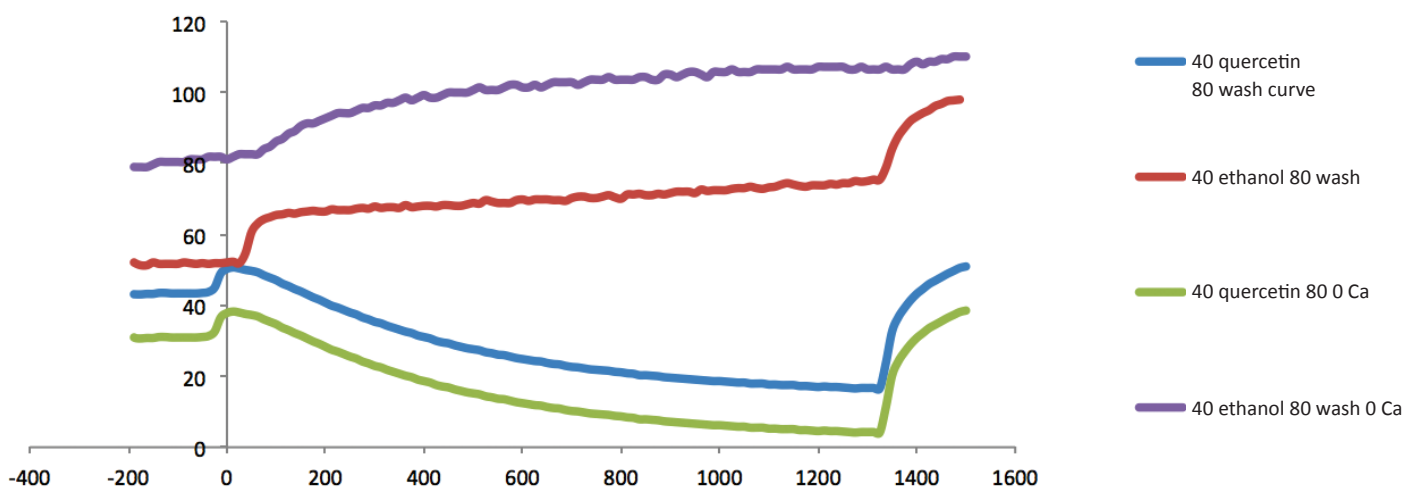
The mechanism that stimulates the  $Ca^{2+}$  release and the molecular mechanism of  $Ca^{2+}$  release (Ny, 1996) from the SR plays an important role in the contraction of porcine pulmonary smooth arterial muscle. The antioxidant quercetin demonstrates an effect on the mechanism of  $Ca^{2+}$  and hence varies the contraction force of the pulmonary arteries. As shown in Figure 6, microdissections of porcine pulmonary arteries, which are in vitro experiments using an isometric muscle bath, were performed to investigate the effects of the antioxidant on the pulmonary arteries. The data achieved using an isometric

force transducer suggest that quercetin reduces the force of contraction in pulmonary arteries both in the presence and absence of  $Ca^{2+}$ .

Keeping the calcium pathway in consideration and observing the data, a model has been proposed. The model predicts that quercetin reduces contractile force by blocking calcium release from the SR. In order to reduce the force of contraction, there needs to be a reduction in  $Ca^{2+}$  influx either from the VOC or from the



**Figure 5.** Contractile force in response to 120 mM KCl stimulation is compared for isolated porcine pulmonary arterial muscle rings in Krebs-Henseleit buffer solution in the presence and absence of calcium. In comparison to the 0  $Ca^{2+}$  Krebs-Henseleit, stimulation in normal Krebs-Henseleit buffer solution resulted in faster force development as noted at 5- to 10-minute early time points.



**Figure 6.** Isolated porcine pulmonary arterial muscle rings (mean diameter = 3 mm) in Krebs-Henseleit buffer solution in the presence and absence calcium when stimulated submaximally to precontract with 40 mM KCl (time = 25 min not shown) varied in their force development (time = 0 min). Here we compare the contractile response of precontracted rings to 1.38 mM ethanol (control) or 60 mM quercetin in ethanol (treatment) (time = 0 min), and then show how the tissue responds to maximal stimulation with 120 mM KCl in the bath to completely depolarize the muscle when 80 mM KCl was added (time = 22 min).



SR. Quercetin in the presence of  $\text{Ca}^{2+}$  shows a higher force of contraction than in the absence of it. This result is demonstrated in Figure 6, where the blue line confirms the fact that extracellular  $\text{Ca}^{2+}$  influx contributes to the higher contraction of PPA, which also is compared to the green line that has no extracellular  $\text{Ca}^{2+}$ . The green line demonstrates a lower force due to the absence of an influx of  $\text{Ca}^{2+}$  via the VOC and quercetin's blockage of  $\text{Ca}^{2+}$  release from the SR. The fact that PPA shows higher contractile force in the presence of quercetin and extracellular  $\text{Ca}^{2+}$  influx compared to the absence of extracellular  $\text{Ca}^{2+}$  indicates that quercetin has no effect on the influx of  $\text{Ca}^{2+}$  through the VOC. If quercetin blocked extracellular  $\text{Ca}^{2+}$  influx through the VOC instead of the SR, then the blue and green lines in Figure 6 would overlap. Since no overlapping occurred, it can be speculated that quercetin blocks  $\text{Ca}^{2+}$  release through the SR.

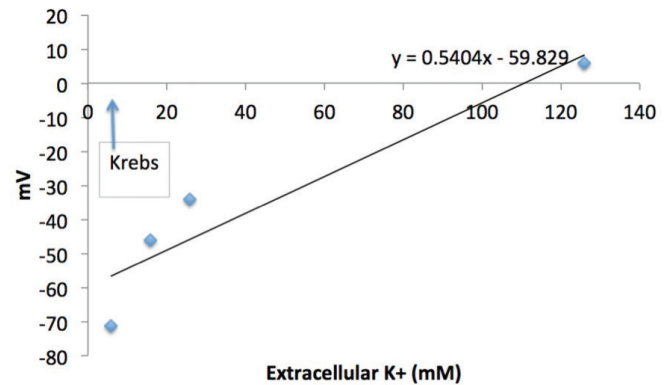
Since the quercetin solution is ethanol-based, the PPA has been contracted in ethanol in the presence and absence of  $\text{Ca}^{2+}$  as a control and labeled as the purple and red lines in Figure 6. These data suggest that quercetin reduces contractile force, because in the absence of quercetin the PPA shows much higher contraction than in the presence of it. However, it is not yet understood why PPA in ethanol in 0  $\text{Ca}^{2+}$  shows a higher contraction than in normal Krebs-Henseleit buffer.

Figure 5 demonstrates the contraction of PPA in response to 120 mM KCl in the presence and absence of  $\text{Ca}^{2+}$ . The data in Figure 2 suggest that even in the absence of  $\text{Ca}^{2+}$ , the PPA muscle shows contraction. The effect of KCl in 0  $\text{Ca}^{2+}$  and the influx of  $\text{Ca}^{2+}$  is not clear. It can be assumed that a  $\text{Ca}^{2+}$  independent signal transduction pathway in response to depolarization with KCl causes the contraction. However, the above results support the hypothesis that quercetin reduces contractile force by blocking  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum  $\text{Ca}^{2+}$  stores in porcine pulmonary smooth arterial muscle, but has no effect on contractile force due to  $\text{Ca}^{2+}$  influx in porcine pulmonary smooth arteries.

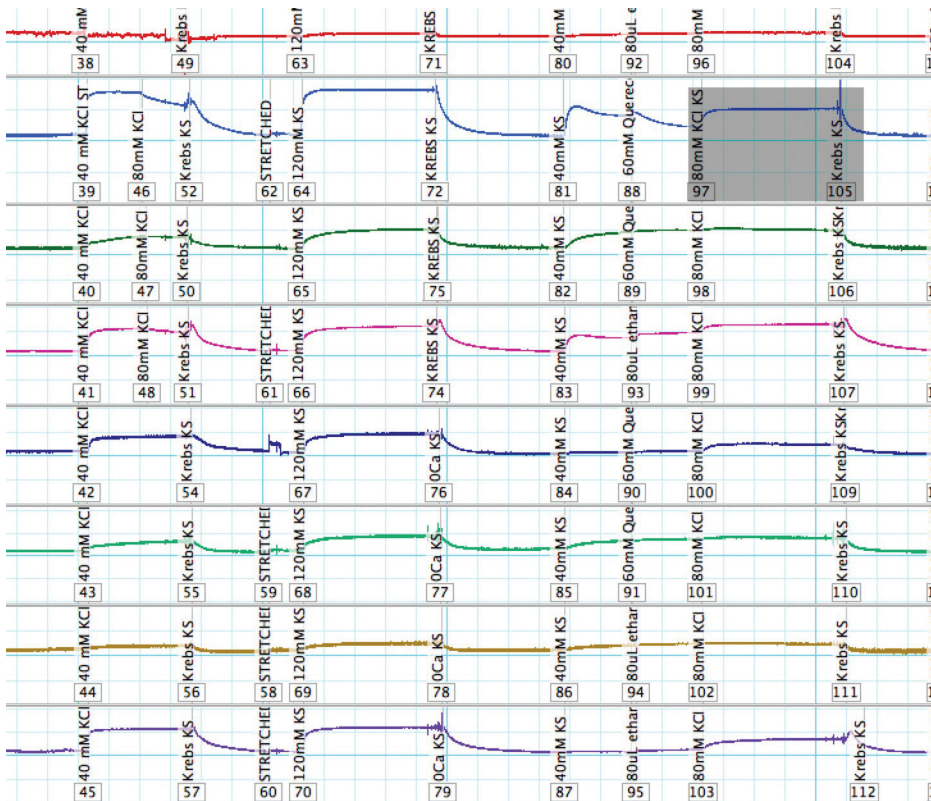
As hypothesized, quercetin exerts cardioprotective effects by reducing contractile force. Similar research on skinned muscle fibers was also performed, where the effect of quercetin on the muscle fibers were analyzed. The results demonstrate that quercetin inhibits the  $\text{Ca}^{2+}$  uptake activities of the SR in skinned muscle fibers but shows no effect on  $\text{Ca}^{2+}$  release (Shoshan, 1980). Therefore, it shows that quercetin does have an effect on  $\text{Ca}^{2+}$  release and uptake by SR, which makes our research relevant and significant.

Hypertension is a complex heterogeneous disorder that can coexist with other cardiovascular abnormalities. Quercetin could hold a potential solution for curing the side effects of hypertension, since it has shown reduction in force of contraction in the presence and absence of  $\text{Ca}^{2+}$ .

## APPENDIX



**Figure 7.** The effect of increasing the extracellular potassium concentration in isolated porcine pulmonary arterial muscle rings (mean diameter = 3 mm) in Krebs-Henseleit buffer solution has been determined in the figure above. The equilibrium potential has been determined using the Nernst equation and plotted with respect to extracellular potassium ( $\text{K}^+$ ) concentration. Increase in extracellular  $\text{K}^+$  increases the equilibrium potential. At  $\text{K}^+$  concentration lower than 110 mM,  $V_m$  demonstrates an acceptably negative voltage, when the L-type VOCs will be closed and  $\text{K}^+$  channel opening will induce relaxation. Increasing  $V_m$  will open the VOCs and allow the extracellular  $\text{Ca}^{2+}$  to flux into the cell, inducing depolarization. A better understanding of equilibrium potential can be obtained using Goldman equation, which is  $E_{ions} = \frac{RT}{F} \ln \left( \frac{P_K [K^+]_o + P_{Na} [Na^+]_o + P_{Cl} [Cl^-]_o}{P_K [K^+]_i + P_{Na} [Na^+]_i + P_{Cl} [Cl^-]_i} \right)$ , where  $P$  defines permeability,  $[K^+]_o$  defines extracellular concentration of  $\text{K}^+$ , and  $[K^+]_i$  defines internal concentration of  $\text{K}^+$ . This equation takes the concentration of the most important ions and its relative permeability into account. Permeability differs due to intravascular pressure, different muscle types, and also different organisms. Therefore, it varies the resting potential in a particular cell. Consequently, we do not know if the Nernst equation is demonstrating appropriate results for porcine pulmonary arterial muscles, but it is a very close approximation.



**Figure 8.** This figure shows the digital recording of the force of contraction of the PPA by force transducer with respect to time.

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