VERTEBRATE ANIMAL SUBJECTS

Aim 1 requires isolation of primary cells from vertebrate animals. Aim 2 of this proposal requires *in vivo* experiments.

1. **Description of Procedures.** 6-7 week old Sprague Dawley rat pups will be used for the cell culture studies in the proposed aims. 10-12 week-old C57BL/6J mice will be used for the *in vivo* experiments. Male animals will be used for all experiments because the I/R phenotype is unreliable and weaker in female mice, compromising statistical power. Further, all power analyses were conducted using literature values from experiments on male mice; no other suitable data were available. For future work, outcomes in female mice will be explored.

   TOTAL RATS: 6 RATS (Sprauge Dawley rats)
   TOTAL MICE: 164 MICE (140 wild-type C57BL/6J mice, 24 C57BL/6J mice expressing firefly luciferase (fLuc; The Jackson Laboratory, No. 025854).

   Aim 1. Number of rats (6 rats): Primary cardiac myocytes will be isolated from 6-7 week old Sprague Dawley rats using the Worthington Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lakewood, NJ). Hearts will be excised from rats deeply anesthetized with isoflurane. 2 rats are required for each experiment. As three cell culture experiments are proposed, 6 total animals are required.

   Aim 2. Number of mice (164 mice):

   Experiment 1 (60 mice): 10-12 week-old C57BL/6J mice will be used to assess the therapeutic benefit of the proposed approach. Power analysis indicates that 15 animals will be required for each of the four treatment groups (vehicle-sham, vehicle-I/R, caged compound-sham, and caged compound-I/R). To perform the surgeries, mice will be intubated and ventilated with 2% (v/v) isoflurane supplemented with oxygen (2 L min^{-1}). After shaving the chest, a thoracotomy will be made at the fourth intercostal space. The pericardium will be dissected, and the LAD will be visualized. 8–0 polypropylene suture will be passed around the artery. Sham surgery will stop at this point (without tightening the ligature). A loose double knot will be tied, and a short length of PE-10 tubing will be placed. The loop will be tightened and secured with a slipknot. Occlusion will be confirmed by confirming pallor in the LAD's territory. The skin will be clamped together and ischemia will be maintained for 60 minutes. Afterwards, the knot will be untied and the tubing removed. The suture will be left in place for determination of infarct size and area-at-risk (AAR) by triphenyltetrazolium chloride (TTC) and Alcian blue staining. After 24 hours of reperfusion, the animals will be reanesthetized and the chest reopened. Analysis of the infarct will be completed after intracardiac injection of potassium chloride and excision of the heart.

   Experiment 2 (68 total mice; 24 commercially-available transgenic mice expressing fLuc in all tissues + 44 wild-type): First, our bioluminescent H_2O_2 probe (caged or active/uncaged) will be injected into commercially-available C57BL/6J mice globally expressing firefly luciferase (fLuc) under the CAG promoter (The Jackson Laboratory, No. 025854). We will validate the genotype in-house using PCR. Mice will be treated with I/R or sham surgery as described above. After injecting the probe intraperitoneally (i.p.; 50 μL of 10 mM solution in 1:1 DMSO:PBS), sedated mice will be imaged using an IVIS 200 *in vivo* bioluminescence imaging system for 40 minutes. Power analysis indicates that 6 animals will be necessary for each of the 4 treatment groups (or 24 total). Second, a 3 $\times$ 2 design will be used (I/R or sham-operated $\times$ vehicle, caged compound, or active compound) to assess the off-target reductive

stress phenotype by qRT-PCR and Western blot. Based on power analysis, 11 animals will be needed for each of the 6 treatment groups (66 mice), but since mice will be carried forward from Experiment 1 to populate the caged compound-I/R and vehicle-I/R treatment groups, only 44 additional mice will be necessary. Therefore, this experiment will require 68 total mice. 24 will be C57BL/6J mice expressing fLuc, and 44 will be wild-type.

Experiment 3 (36 mice): To determine the extent to which the proposed treatment approach reduces chronic injury after ischemia-reperfusion, mice will be monitored for 28 days post-surgery (see above for a description of the I/R and sham operation). At five time points (baseline, 3 days, 7 days, 14 days, and 28 days), the mice will be anesthetized for echocardiography. After the final echocardiographic reading, anesthesia will be deepened, and hemodynamics will be assessed by PV catherization inserted via the carotid artery (or — as an alternative approach — via the open chest). Tissue histology and angiogensis will then be characterized. Based on power analysis and compensating for attrition (to ensure that the sample size is large enough for adequate power by the end of the experiment), 9 animals per treatment group will be necessary. Mice will be randomized into four treatment groups (vehicle, acute treatment, chronic treatment, and acute plus chronic treatment), necessitating 36 animals total.

2. **Justifications.** Live organisms are necessary for the proposed work because cell culture experiments alone do not accurately recapitulate myocardial ischemia-reperfusion in a way that allows assessment of clinically-important parameters (infarct size/area-at-risk and hemodynamics). Additionally, cultured cells cannot be used to detect perturbations of cell signaling pathways in uninjured organs. Current mathematical models and computer simulations are insufficient for this purpose. Accepted myocardial I/R models are not available in lower species (e.g., invertebrates). Sprague Dawley rats were chosen because they have been well-established as a model organism for myocardial ischemia-reperfusion and myocyte hypoxia/reoxygenation studies, and are widely considered to be a docile, easy-to-handle strain. C57BL/J mice were chosen due to the strain's robustness and well-validated history of use with our model. The PI has completed all required University of Pittsburgh EH&S training and will handle all chemicals and biological materials in accordance with standard operating procedures.

3. **Veterinary Care.** Animals will be housed at the Division of Laboratory Animal Resources (DLAR) at the University of Pittsburgh. Rats will be housed in pairs and mice 4–5 per cage with unrestricted access to food and water, and with bedding provided in each cage. Staff veterinarians and veterinary technicians in addition to the PI will monitor the animals' health at least once per day; staff will recommend treatment with antibiotics and analgesics as necessary to minimize pain in the animals. Veterinary staff members are available during business hours, and are always on-call in case of emergencies. The staff can also intervene or suggest euthanasia for any animals showing signs of distress, which, for this model, include hunched, immobile posture; spiked coat; self-mutilation; aggressive behavior; vocalizations; and signs of excessive inflammation at the surgical site. All mice will be handled according to the regulations of the American Association for the Accreditation of Laboratory Animal Care. University of Pittsburgh Medical Center is a member in good standing with this organization and strictly adheres to all regulations for the welfare and humane treatment of animals.

4. **Minimization of Pain and Distress.** For surgical procedures, anesthesia with isoflurane will be accompanied by analgesia and sedation with ketamine and xylazine (100 mg kg^{-1} i.p. and 10 mg kg^{-1} i.p., respectively). Anesthesia will be induced using 2-5% isoflurane in oxygen and maintained using 2% isoflurane in oxygen. After surgery, EMLA cream will be applied to

the surgical site as a local anesthetic. Post-operative animals will recover in an oxygen-rich chamber, and will not be returned to their housing until fully awake and mobile. Buprenorphine hydrochloride (0.1 mg kg^{-1} SC BID) and ketorolac (5 mg kg^{-1} SC BID) will be given for three days to minimize pain. Antibiotics will be given if the surgical site becomes infected. This surgery has a risk of pneumothorax, and animals will be euthanized if discomfort arises from this condition. Buprenorphine will be given as needed if any signs of pain or distress are observed in the animals. If any animals have a severe negative reaction to surgery, characterized by aggression, vocalization, and/or hunched posture, veterinary staff will be consulted, and animals will be euthanized if necessary.

5. **Euthanasia.** Euthanasia will be performed by cardiac injection of potassium chloride (KCl) in deeply anesthetized animals. This method is necessary to ensure that the heart is in diastole when it is excised, which is essential for the proposed experiments. The American Veterinary Medical Association (AVMA) *Guidelines for the Euthanasia of Animals* approves this method as a special case if the animal is fully anesthetized. Isoflurane will be used to ensure full anesthesia before KCl injections.