

Abstract

Background: Ischemia and reperfusion (I/R) contribute to myocardial dysfunction through activation of the protease calpain. We have reduced cardiac dysfunction in a porcine model of I/R by inhibition of calpain activity. The hypothesis is that calpain regulates cardiac inflammatory and vasoactive proteins through a nuclear factor-kappaB (NF-κB)-dependent pathway. **Methods:** Cardiomyocytes isolated from neonatal rats were subjected to I/R by placing cells in 0.5% oxygen culture media without glucose for 18 hours then reperfusion in glucose-containing media at 21% oxygen for 6 hours. Calpain inhibitor (2, 10, and 50 μM, Z-Leu-FMK) or vehicle was added 30 minutes prior to ischemia. Endothelin-1 was measured in culture media by ELISA and NF-κB (p50 and p65) activity by ELISA of nuclear extracts. Nitric oxide (NO) release into media was directly measured by electrochemical sensor. **Results:** Ischemia and I/R increased endothelin-1 production by untreated cardiomyocytes (Table 1). Calpain inhibition lowered media endothelin-1 in ischemic and I/R cultures in a dose-dependent manner to levels similar to normal cultures. Calpain inhibition increased NO production in normal cells to levels similar to I/R. Calpain inhibition during I/R decreased NF-κB activity in cardiomyocyte nuclear extracts. **Conclusions:** Calpain-regulated cardiac dysfunction following I/R might be mediated by NF-κB-dependent inflammatory and vasoactive proteins including endothelin-1 and NO.

Introduction

Congenital heart disease is the number one cause of death from birth defects in the first year of life. Many complex congenital heart defects result in cyanotic children that undergo surgical repair in the first few weeks of life. These children often require cardiopulmonary bypass and deep hypothermic cardiac arrest (CPB/DHCA) in order to allow adequate visualization for repair. The resultant myocardial circulatory arrest generates an ischemia/reperfusion injury that has been associated with the generation of cardiopulmonary dysfunction in the postoperative period. Multiple attempts have been made to mitigate this dysfunction by administering myocardial protectors during ischemia, but have been met with limited success.

Calpain, a ubiquitous intracellular protein with known calcium-dependent cysteine protease activity, has been implicated in the pathogenesis of multiple disease processes including cardiopulmonary dysfunction following CPB. Evidence suggests that CPB/DHCA may generate cardiopulmonary dysfunction through an ischemia/reperfusion injury that can affect intracellular calcium handling and the activation of calpain. Calpain is known to degrade several targets within the cell, including cytoskeletal proteins, membrane receptor proteins, and various enzymes. Inhibitory proteins, as well as proteins involved in excitation coupling, may also be targeted by calpain. The activation of calpain may then significantly affect cellular physiology and function, which suggests that increased levels or altered regulation of calpain leads to loss of cardiopulmonary function. Therefore, the inhibition of calpain activity with synthetic calpain inhibitors may alleviate dysfunction seen following CPB/DHCA. Figures 1 & 2 indicate the improvement in cardiopulmonary function in a neonatal piglet model of CPB/DHCA. Pulmonary vascular resistance was reduced and oxygen delivery was maintained 120 min after CPB/DHCA.

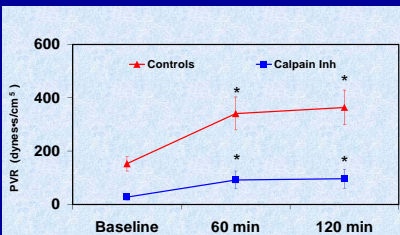


Figure 1. Pulmonary Vascular Resistance (PVR). Calpain inhibitor prevented the rise in pulmonary vascular resistance seen in control piglets after CPB/DHCA (*p<0.05 vs. baseline).

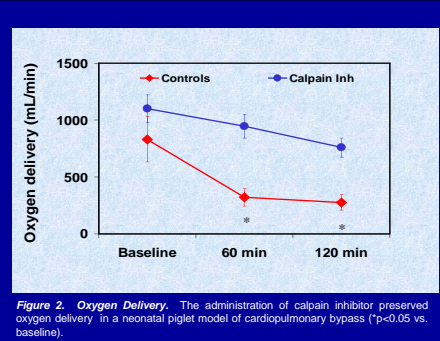


Figure 2. Oxygen Delivery. The administration of calpain inhibitor preserved oxygen delivery in a neonatal piglet model of cardiopulmonary bypass (*p<0.05 vs. baseline).

Methods

Hearts are aseptically removed from Sprague-Dawley neonatal rats, 1-2 days of age, to ice-cold buffer. Ventricles are dissociated with pancreatin and collagenase from Worthington Biochemical (Freehold, NJ) in a Wheaton stir flask for 20 minutes. Released cells are removed and mixed with FBS to stop digestion then resuspended in plating medium. This procedure is repeated 9-10 times. Non-muscle cells are minimized in cultures by preplating for 70 minutes in M199, 15% fetal bovine serum, and antibiotics in an atmosphere of 5% CO₂ and 95% air. After preplating, suspended and loosely attached cells are collected and plated on gelatin-coated plates at 2.5x10⁶ cells/cm² for 24 hours to allow attachment. Plating medium is then replaced with serum-free medium for 24 hours. Cardiomyocytes are identified by α-sarcomeric actin immunocytochemistry on random plates and have previously resulted in less than 5% non-myocyte contamination. Experiments are initiated 48 hours after isolation. Control normoxic cells are cultured in DMEM with 4.5 g/L glucose, 2% FBS and antibiotics in a humidified atmosphere of 5% CO₂ and 95% air. Ischemia and reperfusion are simulated by culturing cells in DMEM as described, except media has no glucose, in an atmosphere of 5% CO₂, 0.5% O₂, and the remainder nitrogen for 18 hours in a hypoxic cell culture chamber (Bioprocess Corp). CO₂ and O₂ are independently regulated within the chamber. After 18 hours of hypoxic conditions, the culture media is collected and cells are rapidly harvested by scraping into deoxygenated detergent lysis buffer. Other cultures are reperused by replacing the hypoxic media with normoxic DMEM containing glucose and continuing culture in 5% CO₂ and 95% air for an additional 6 hours. Normoxic control cells are also harvested after 18 hours and at each specified reperfusion time point for comparison. Dose response experiments are repeated at least three times. Protein expression of genes important in mediating inflammation are measured in the cell lysates or culture media. Experiments with calpain inhibitor use the synthetic peptide inhibitor Z-Leu-Leu-Tyr-FMK, which is cell permeable and allows for direct comparison to in vivo studies.

Treatment groups (n=4) include:
a) Control cells with and without simulated ischemia and reperfusion. These groups establish effects of simulated ischemia and reperfusion.
b) Addition of a calpain inhibitor (2, 10, and 50 μM, Z-Leu-Leu-Tyr-FMK) 30 minutes prior to hypoxia. Calpain inhibitor is expected to maintain IκBα protein levels in the cytosol. NF-κB activation is anticipated to be lower than control cells, but not abolished. The proteins of inflammatory markers regulated by NF-κB are expected to be down-regulated.

Endothelin-1 Analysis: A commercial endothelin-1 (ET-1) immunoassay kit (R & D Systems, Minneapolis, MN) was used to measure ET-1 concentration in the rat neonatal cardiomyocyte (RNCM) media.

Protein Analysis: Protein levels were determined by commercial western blot analysis (Invitrogen, Carlsbad, CA). Cells were lysed in a detergent lysis buffer (50 mM Tris, 150 mM NaCl, 0.2% NaN₃, 0.1% NP-40). Western blots were performed with 30 μg of total protein separated on 4-12% acrylamide bis-tris gels (Invitrogen, Carlsbad, CA) by SDS-PAGE. Immunoblots were probed with antibodies for desired protein (calpain I, IκBα, and IL-6), as well as α-sarcomeric actin and GAPDH. Secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit, anti-mouse, or rabbit anti-goat IgG. Proteins were visualized with the Western Breeze chemiluminescent detection system according to the manufacturer's instructions (Invitrogen). Protein levels are reported as a ratio of target protein to α-sarcomeric actin levels on the same immunoblot to correct for background.

Statistical Analysis of the Data: Comparisons between treatments are made by unpaired Student's t-test with p<0.05 considered significant. Analyses are conducted with Statview 4.01 software (Abacus Concepts, Berkeley, CA).

Results

	Untreated				Calpain Inhibitor (2 μM)				Calpain Inhibitor (10 μM)				Calpain Inhibitor (50 μM)			
	Nor	Ischemia	Nor	I/R	Nor	Ischemia	Nor	I/R	Nor	Ischemia	Nor	I/R	Nor	Ischemia	Nor	I/R
NO (nM)	190±40	231±52	199±28	231±48	220±49	249±53	247±49 b	261±33	235±34	271±54	276±35 b	272±32	261±45b	276±25	283±36b	280±39
ET-1 (pg/mL)	1.8±.5	2.7±.3a	2.7±.5	4.5±.9a	1.5±.4b	3.0±.3	2.0±.4b	4.1±.8	1.6±.4	2.4±.6	2.2±.6	3.7±.11	1.5±.4	1.8±.4b	1.6±.7	2.7±1.2a,b

Table 1. Cardiomyocyte Response to Ischemia and Reperfusion ^aP<0.05 vs. normal cells at same time point within the same treatment. ^bP<0.05 vs. untreated at same time point means±standard deviations

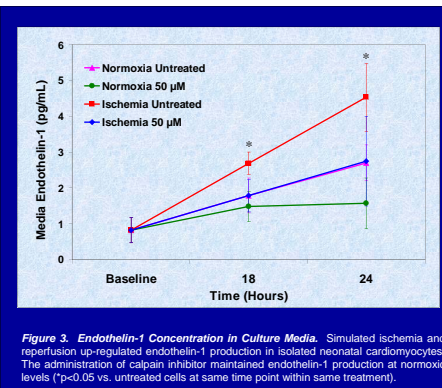


Figure 3. Endothelin-1 Concentration in Culture Media. Simulated ischemia and reperfusion up-regulated endothelin-1 production in isolated neonatal cardiomyocytes. The administration of calpain inhibitor maintained endothelin-1 production at normoxic levels (*p<0.05 vs. untreated cells at same time point within same treatment).

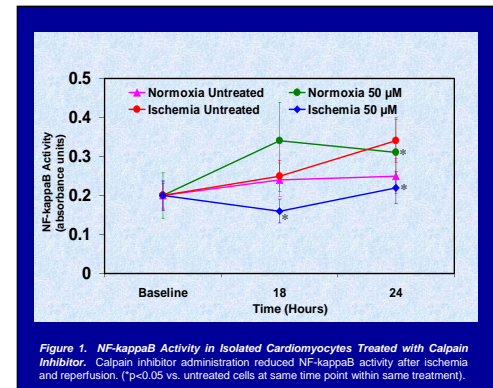


Figure 4. NF-kappaB Activity in Isolated Cardiomyocytes Treated with Calpain Inhibitor. Calpain inhibitor administration reduced NF-kappaB activity after ischemia and reperfusion. (*p<0.05 vs. untreated cells at same time point within same treatment).

Summary

- Levels of the vasoconstrictor endothelin-1 in ischemic and reperused culture media were maintained at levels comparable to normoxic counterparts with the addition of calpain inhibitor.
- Calpain inhibitor (50 μM) increased the production of the vasodilator nitric oxide in normoxic cultures of isolated cardiomyocytes. Inhibitor treatment did not further elevate the increase detected with ischemia and reperfusion.
- The activity of NF-kappaB, a transcription factor that mediates endothelin-1 and nitric oxide expression, is up-regulated with reperfusion following ischemia. Calpain inhibitor alleviates the rise in NF-kappaB activity associated with reperfusion injury.
- Calpain-regulated cardiac dysfunction following ischemia and reperfusion might be mediated through NF-kappaB-dependent inflammatory mediators and vasoactive factors.

Disclosures

This study was supported by NIH R01 HL077653 to JYD. There are no conflicts of interest to report by the authors.