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Effects of selective cyclooxygenase-2 and non-selective COX inhibition on myocardial function and perfusion

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Abstract

Non-selective NSAIDs and selective cyclooxygenase-2 (COX-2) inhibitors are purported to increase adverse cardiovascular events. We hypothesized COX-2 inhibitors would alter myocardial blood flow, microvascular reactivity, oxidative stress, and prostaglandin levels. Adult Yorkshire swine were divided into three groups: no drug (control, n=7), a non-selective COX inhibitor (naproxen 400mg daily, NAP, n=7), or a selective COX-2 inhibitor (celecoxib 200mg daily, CBX, n=7). After 7 weeks physiologic measurements were taken and tissue harvested. Animals in the CBX group demonstrated significantly higher blood pressure and rate pressure product. The NAP and CBX groups demonstrated an increased microvascular contraction response to serotonin. The NAP group showed increased myocardial levels of thromboxane and lower levels of prostacyclin. Levels of protein oxidative stress were increased in the CBX group. Myocardial apoptosis was lowest in the NAP group. Immunoblotting demonstrated decreased VEGF and phospho-eNOS expression in the NAP and CBX groups. Myocardial TNF α was increased in both the NAP and CBX groups. Immunostaining for thromboxane A₂ synthase and receptor demonstrated expression within the vascular smooth muscle and no observable differences between groups. Non-selective and selective COX inhibition does not alter myocardial perfusion, but results in altered myocardial and vascular physiology that may have implications regarding cardiovascular risk.

Keywords

Myocardial physiology; Cyclooxygenase inhibition; Prostaglandins; Microvascular reactivity

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Introduction

Cyclooxygenase (COX) inhibitors specific for the inducible isoform (COX-2) of the enzyme were introduced in the early 1990's. This class of drug was developed to reduce the incidence of gastrointestinal complications associated with the use of non-specific non-steroidal anti-inflammatory drugs (nsNSAIDs). In a number of clinical trials COX-2 inhibitors were demonstrated to have an improved gastrointestinal safety profile.¹ In the analysis of the VIGOR trial it was observed that patients with rheumatoid arthritis treated with the COX-2 inhibitor rofecoxib arm were at increased risk of myocardial infarction and stroke as compared to patients taking naproxen.²

In 2004 Merck voluntarily removed rofecoxib from the market after the APPROVe trial demonstrated a two fold increase in the risk of myocardial infarction in patients taking 25 mg of the drug after 18 months.³ The resulting scrutiny of ongoing trials showed that the risk of cardiovascular adverse events was not limited to rofecoxib, and most selective COX-2 inhibitors and nsNSAIDs were implicated.^{1, 4-6} At this time both selective and non-selective NSAIDs carry a black box warning from the US food and Drug Administration regarding increased cardiovascular risk.⁷ Further study has revealed that the risk for most COX-2 inhibitors and nsNSAIDs is dose-dependent.⁸

In 2007, the American Heart Association published a position paper on the use of NSAIDs and recommended a limited role for selective COX-2 inhibitors.⁹ However, most clinical trials examining the incidence of cardiovascular events in patients taking either selective COX-2 inhibitors or nsNSAIDs have found no difference in the incidence of adverse events.^{1, 4, 10}

The leading theory as to why selective COX-2 inhibition may increase cardiovascular events was proposed as a result of a study by FitzGerald, and relates to differential levels of opposing prostaglandins.¹¹ It has been hypothesized that selective COX-2 inhibition will lead to decreased endothelial prostacyclin (PGI₂), a prostaglandin with vasodilatory and anti-thrombotic properties, and increased levels of thromboxane (TXA₂), a platelet-derived pro-thrombotic vasoconstrictor. This shift in the myocardial prostaglandin milieu is thought to lead to increased coronary thrombosis and myocardial infarction (MI). This hypothesis was supported in a study of healthy volunteers given escalating doses of a COX-2 inhibitor (celecoxib)¹¹. A nsNSAID (ibuprofen) suppressed TXA₂-dependant platelet aggregation and celecoxib did not. Urinary concentrations of PGI₂ were decreased equally by both drugs, but TXA₂ concentrations were not reduced in the celecoxib group.¹¹

To date there have not been any studies examining the effects of nsNSAIDs and COX-2 inhibitors under baseline (non-ischemic) conditions in a clinically relevant large animal model. In this study our aim was to further define the effects of NSAIDs on the non-ischemic myocardium. We hypothesized that nsNSAIDs and COX-2 inhibitors would influence myocardial perfusion, microvascular reactivity, prostaglandin levels, and the angiogenic potential of the myocardium.

Methods

Study Design

Yorkshire miniswine (Parsons Research, Amherst, MA) were divided into three groups. One received celecoxib 200 mg orally per day (CBX, n=8), another received naproxen 400 mg orally per day (NAP, n=10), and the third received no drug (control, n=8). All animals received drug treatment for seven weeks.

After seven weeks of drug therapy swine were anesthetized, the heart was exposed, and physiologic measurements were taken, followed by euthanasia. For the surgical procedure, anesthesia was induced with ketamine (10 mg/kg IM) and thiopental 2.5%, and maintained with a gas mixture of oxygen at 1.5 - 2 L/min and 3.0% isoflurane. The animals were intubated and mechanically ventilated at 16 breaths/min. After the heart was harvested, myocardial samples were rapidly frozen in liquid nitrogen (molecular studies and immunohistochemical studies) or placed in 4°C Krebs solution (microvessel reactivity studies).

All experiments were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Animals were cared for in compliance with the Harvard Medical Area Institutional Animal Care and Use Committee and in accordance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' (NIH publication no. 5377-3 1996).

Measurement of Global and Regional Myocardial Function

Indices of global and regional left ventricular (LV) function were obtained prior to animal sacrifice for 10 sequential beats using a Sonometrics system (Sonometrics Corp. London, ON, Canada), as previously described.¹² Blood pressure was measured with an intra-arterial pressure catheter placed in the aorta.

Myocardial Perfusion Analysis

Myocardial perfusion was determined during each procedure with isotope-labeled microspheres (ILMs), 15 µm diameter (BioPAL Worcester, MA) using previously reported methods.¹³ Briefly, 1.5×10^7 Lutetium (rest) and Europium (pace) labeled ILMs were injected during the procedure. Following euthanasia, left ventricular sections were collected for ILM assay. The samples were exposed to neutron beams and microsphere densities were measured using a gamma counter.

Microvessel Studies

After cardiac harvest, coronary arterioles (80-180µm in diameter) from the myocardium supplied by the left anterior descending coronary artery (LAD) were dissected from the surrounding tissue and placed in an isolated microvessel chamber as described previously.¹⁴ Response to the vasoconstricting agent serotonin (5-HT, 10^{-9} mol/L to 10^{-5} mol/L) was assessed in non-precontracted vessels. Next, the microvascular response to vasorelaxing agent adenosine diphosphate (ADP, 10^{-9} to 10^{-4} mol/L, an endothelium dependent vasodilator) and sodium nitroprusside (SNP, 10^{-9} to 10^{-4} mol/L, an endothelium independent vasodilator) were evaluated after precontraction of microvessels by 30-50% of the baseline diameter with the thromboxane A₂ analog U46619 (1.0 µM). Responses were defined as percent constriction of baseline diameter for serotonin and SNP, and percent relaxation of the precontracted diameter for ADP. All drugs were applied extraluminally. Responses were defined as percent constriction of baseline diameter. All reagents were obtained from Sigma-Aldrich (St Louis, MO).

Tissue Thromboxane and Prostacyclin Levels

Tissue levels of stable thromboxane and prostacyclin breakdown products, 11-dehydrothromboxane B₂ (11-d-TXB₂) and 6-keto prostaglandin F₁α (6-k-PGF₁α), respectively were measured by ELISA (Neogen Corp, Lexington, KY). Tissue lysates underwent liquid-liquid exchange, extraction, and concentration according to the manufacturer's recommendations. The samples were put into an antibody coated plate along

with HRP conjugated 6-k-PGF1 α . The plate was washed and HRP substrate added. The plate was incubated and read on at 650 nm, and sample results were plotted against the standard curve.

Serum Prostacyclin Levels

Levels of 6-k-PGF1 α were measured by ELISA (Neogen Corp). Serum samples underwent liquid-liquid exchange, extraction, concentration, and assay as described above.

Immunoblotting Studies

Sixty micrograms of total protein was fractionated by 4-20% gradient, SDS polyacrylamide gel electrophoresis (Invitrogen, San Diego, CA) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Each membrane was incubated with specific primary antibodies (Cell Signaling, Beverly, MA). Immune complexes were visualized with the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Bands were quantified by densitometry of autoradiograph films. Ponceau staining was used to ensure equal protein loading.

Immunofluorescence Studies

Frozen sections (12 μ m in thickness) of myocardium from the left ventricle were formalin fixed. Antibodies against proliferation marker Ki-67 (Abcam Inc., Cambridge, MA) and endothelial marker PECAM-1 (CD-31, R&D Systems, Minneapolis, MN) were simultaneously applied to the sections and incubated. Arteriolar density was assessed by staining with smooth muscle actin (SMA, Cell Signaling). Thromboxane synthase (TS, Abcam) and receptor (TR, Cayman Chemical, Ann Arbor, MI) were assessed by co-staining with SMA. Detection was obtained using appropriate secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Sections were mounted in Vectashield with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Photomicrographs were taken with a Zeiss Axiolab microscope (Carl Zeiss Inc, Thornwood, NY) equipped with a digital camera at 200 \times magnification (Photodoc, Upland, CA). Dividing endothelial cells, capillary density, and arteriolar cell density were counted in a blinded fashion. Data are presented as number of positive cells/mm².

Myocardial Protein Oxidative Stress

Dinitrophenylhydrazine-derivatized myocardial tissue homogenates were separated by 10% polyacrylamide gel electrophoresis and transferred to PVDF membranes (Chemicon International, Inc. Temecula, CA). The membranes were incubated with primary antibody specific to dinitrophenylhydrazine, followed by incubation with a horseradish peroxidase-linked secondary antibody. Immune complexes were visualized with the enhanced chemiluminescence detection system (Amersham).

Quantification of Apoptotic cells

Apoptotic cells in the myocardium were identified using the ApopTag detection kit according to manufacturer's specifications (Chemicon Inc.). At least one cm² of tissue was analyzed from each animal (4 per group). The slides were counter-stained with hematoxylin allowing the morphologic identification of cell types, specifically in distinguishing cardiomyocytes from other cells. The number of TUNEL-positive cardiomyocytes is expressed as positive cells/cm².

Data Analysis

All results are expressed as mean \pm SEM. Microvessel responses are expressed as percent relaxation of the precontracted diameter, or contraction of the baseline diameter, and were

analyzed using two-way, repeated measures analysis of variance (ANOVA) with a post-hoc Bonferroni test, which was applied to interactions of treatment and dose. Western blots were analyzed after digitalization (ScanJet 4c; Hewlett-Packard, Palo Alto, CA) with NIH ImageJ 1.33 software (National Institute of Health, Bethesda, MD). For data analysis, levels of phosphorylated proteins were normalized to total expression levels. Comparisons between the three groups were analyzed by one-way, repeated measures ANOVA with Newman-Keuls Multiple Comparison post-hoc test, using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA).

Results

Functional

Both systolic and diastolic blood pressures were significantly increased in the CBX group as compared to the control and NAP groups. Calculation of rate pressure product (RPP) demonstrated significantly lower values in the NAP group as compared to the control and CBX groups. There was no difference in RPP between the control and CBX groups (Table 1). The weight of the animals increased similarly among the groups from the initiation of drug treatment until the end of the study (increase in control 10.1 ± 1.6 kg, naproxen 11 ± 1.2 kg, and celecoxib 7.7 ± 0.6 kg, $p = 0.18$).

Myocardial Perfusion

Myocardial perfusion was assessed by microsphere density. There was no difference in blood flow to the myocardial territory supplied by the LAD at rest or with pacing between the groups (Table 1).

Microvascular responses

There was no difference in the baseline diameter of the microvessels between groups (control 135 ± 9 μm , NAP 128 ± 14 μm , and CBX 144 ± 13 μm , $p = 0.67$), or the precontracted diameter of the microvessels when the responses to ADP or sodium nitroprusside were examined (control 95 ± 6 μm , NAP 77 ± 8 μm , and CBX 96 ± 7 μm , $p = 0.16$). There was no difference in the percent vessel precontraction between the groups with U46619 (control $-37 \pm 2\%$, NAP $-36 \pm 3\%$, and CBX $-34 \pm 1\%$, $p = 0.73$).

The microvessel contraction response to serotonin was significantly increased in both the NAP and CBX groups as compared to the control group. There was no difference between the two drug treatment groups (Figure 1A). There was no difference in microvessel response to the endothelial dependent vasorelaxation agent ADP (Figure 1B). There was no difference in the microvascular response to endothelial independent drug SNP (control $93.4 \pm 5.7\%$, NAP $81.5 \pm 5.4\%$, CBX $74.9 \pm 7.8\%$, $p = 0.25$ at 10^{-5} M).

Prostaglandin levels

The level of 11-d-TXB₂ in the myocardial tissue was significantly increased in the NAP group as compared to the control and CBX groups. There was no difference between the control and CBX groups (Figure 2A). The level of 6-k-PGF1 α was significantly decreased in the NAP group, and there was no difference between the control and CBX groups (Figure 2B).

The serum levels of 6-k-PGF1 α were markedly decreased in NAP and CBX groups as compared to the control (control 3457 ± 770 ng/mL, NAP 281.8 ± 81.5 ng/mL, CBX 105.6 ± 81.6 ng/mL, $p < 0.001$).

Immunofluorescence

Both the NAP and CBX groups had fewer myocardial arterioles than the control group (Figure 3A-D). There was a trend toward lower capillary density in the myocardium of NAP and CBX groups, but this did not reach significance (Figure 3E). The number of dividing endothelial cells, positive staining for both CD-31 and Ki67, was decreased in the NAP and CBX groups as compared to the control group (Figure 3F).

Protein Expression

There was a significant downregulation of the angiogenic protein VEGF in the NAP and CBX groups as compared to the control group (Figure 4A). There was no difference in fibroblast growth factor (FGF) between the groups ($p=0.14$) (not shown). The potent vasodilator and pro-angiogenic protein phospho-eNOS was significantly downregulated in the drug treatment groups (Figure 4B). There was an increase in the myocardial expression of the inflammatory cytokine TNF α in both the NAP and CBX groups as compared to the control group (Figure 4C). Levels of activated Akt and ERK (phospho-Akt (thr308) and phospho-ERK (thr218/tyr220)), normalized to total protein expression levels, were increased in the NAP group when compared to the control and CBX groups (Figure 4D and E).

Protein Oxidative Stress Levels

The levels of protein peroxidation were increased in the CBX as compared to the control and NAP groups. There was no difference between the control and NAP groups (Figure 5A).

Levels of Apoptosis

The level of apoptosis in the myocardium at the time of tissue harvest was lowest in the NAP group. There was no difference in the number of TUNEL positive cells between the control and CBX groups (Figure 5B).

Discussion

NSAIDs have been purported to increase the risk of adverse cardiovascular events in patients. Although this has been described in a number of large clinical trials, other trials have found both non-selective and selective COX-2 inhibitors to be safe. The potential underlying basic pathophysiology and molecular influences of the purported increased incidence of adverse events with COX inhibition have not been clearly defined. Previous studies in cultured cell models have suggested that NSAIDs will impact the cardiovascular system.^{15, 16} We sought to define the effects of these drugs on the cardiovascular physiology, prostaglandin levels, and the angiogenic potential in a large animal model that mimics human cardiovascular physiology in most respects. There were a number of significant findings in this work that may give insight into how nsNSAIDs and selective COX-2 inhibitors affect the myocardium. Some of the effects are similar and others are divergent between the groups. Celebrex led to increased blood pressure and protein peroxidation, while naproxen caused a decrease in levels of prostacyclin and an increase in thromboxane, as well as decreased apoptosis. Both drugs altered the microvascular contractile response to serotonin, decreased serum PGI₂ levels, and diminished vascular capillary density in this model during which the cardiac weight increases considerably during the course of the experiment. However, in the present study, myocardial perfusion surprisingly was not significantly affected by treatment with either naproxen or celecoxib.

Most NSAIDs have been implicated in causing systemic hypertension. In the current study only the selective COX-2 inhibitor was associated with increased blood pressure. The etiology of NSAID induced hypertension is multifactorial and likely involves retention of sodium, increased renal vascular constriction, and microvascular dysfunction.^{17, 18} The

increased microvascular contraction response to serotonin, and presumably other vasoconstrictive agents, may offer one potential mechanism for the increased blood pressure. In the current work we examined the effect of three molecules on the coronary microvasculature. *In vivo* there are many molecules influencing microvascular tone, and dysfunction has been linked to the development of hypertension.¹⁹ Additionally, while increased expression of thromboxane receptor can lead to hypertension¹⁴, we did not observe differences in thromboxane receptor or thromboxane synthase in the vascular smooth muscle of either treatment group, nor was the microvascular response to the TXA₂ analog U46619 different between groups.

The decrease in rate pressure product in the NAP group was unexpected. While a decreased RPP may have the potential to decrease cardiovascular risk, the effects of the nsNSAID on levels of prostacyclin could increase risk. According to the FitzGerald hypothesis, the increase in cardiovascular events with selective COX-2 treatment may be due to an imbalance of pro- and anti-thrombotic prostaglandins.¹¹ In our study naproxen treatment led to increased thromboxane and decreased prostacyclin, while the selective COX-2 inhibitor, celebrex, did not, though both drugs significantly decreased the serum levels of prostacyclin. This finding is contrary to what would be predicted by the FitzGerald hypothesis, which would predict that a selective COX-2 inhibitor would produce more TXA₂ and less PGI₂ compared to a non-selective COX inhibitor.

Both naproxen and celecoxib affected molecular and cellular indices of angiogenesis although perfusion was similar between treatment groups. Both drugs decreased arteriolar and to a lesser extent, capillary density in the young pigs undergoing significant myocardial growth during the course of the experiment. In addition, endothelial cell proliferation was diminished by both naproxen and celecoxib as compared to the control. VEGF, phospho-eNOS, and COX-2 are all integral parts of the angiogenic process. The importance of COX-2 as an essential component of angiogenesis has been well described. In one study, the formation of vessels in Matrigel was decreased in endothelial cells transfected with COX-2 silencing RNA, while cells overexpressing COX-2 led to increased vascular density.²¹ Collateral development in ischemic myocardium can bypass coronary vessels affected by occlusive disease. The presence of a well-developed network of collaterals has been associated with a reduced risk of death and myocardial injury after an MI.²³ Recently, we reported that both naproxen and celecoxib alter the vascular and myocardial homeostasis under conditions of chronic myocardial ischemia.²⁴ These changes did not lead to a reduction in collateral dependent perfusion despite diminished capillary density as compared to non-treated control animals. In fact, naproxen was actually associated with a significant increase in collateral dependent perfusion, while celecoxib was associated with no difference in collateral dependent perfusion compared to the non-treatment control. Interestingly, the decrease in arteriolar density did not translate to reduced blood flow to the myocardium in either study, as perfusion at rest and during ventricular pacing was not significantly different between groups.

Vascular density is one determinate of blood flow, but other mechanisms may have compensated including neurohumoral influences and autoregulation. Coronary autoregulation and metabolic control of perfusion are powerful mediators of coronary blood flow that ensure constant blood flow to meet the heart's metabolic demands.²⁵ The mechanism by which autoregulation works is not completely understood, but likely involves vasoactive metabolites such as those listed above and autonomic control of myogenic responses.²⁶ We assessed a limited number of these potential mediators of blood flow, though they likely played a role in maintaining myocardial perfusion. The implications of the above described differences between groups in adult patients not undergoing significant myocardial growth or ischemia are not known.

While myocardial perfusion was not different between groups in this study, levels of protein oxidative stress were increased in the CBX group as compared to the other groups. Increased oxidative stress is often related to an increase in reactive oxygen species (ROS). NSAIDs have been shown in cell culture studies to either increase or decrease ROS. A prolonged increase in ROS can lead to cardiac remodeling and ultimately heart failure via cardiomyocyte hypertrophy, induction of interstitial fibrosis, and apoptosis.²⁷ Upregulation of TNF α has been associated with elevated ROS.²⁸ In our study the reason for elevated TNF α in the drug treatment groups was not clear, though it may have been compensatory for the generally anti-inflammatory action of NSAIDs. While increased ROS and TNF α are associated with apoptosis, surprisingly we did not observe this, and apoptosis at the time of tissue harvest was actually decreased in the NAP group.

Control of apoptosis is complex, but it has been demonstrated that both Akt and ERK decrease this process.²⁹ In a recent study it was shown that phospho-ERK, in association with IL-10, decreases TNF α induced apoptosis.³⁰ The increased expression of these activated proteins may have contributed to the reduced level of apoptosis in the naproxen treated group.

Limitations

There are several limitations of this study. First, in most situations the porcine coronary circulation closely mimics the physiology and pathophysiology of the human coronary circulation, though this may not necessarily be the case for COX inhibition. Second, while we were able to assess a number of physiologic and molecular pathways, we did not measure platelet activity or the production of thromboxane by platelets. Platelet-derived thromboxane will contribute to a pro-thrombotic, vasoconstrictive milieu in the myocardium. Prostaglandins are not only synthesized in vascular tissue, but also in the lung, gut, prostate gland and other organs, which may affect the conclusion that can be drawn from this study. Finally, only a single dose of each drug was investigated, and different dosages would likely result in different effects on the myocardium. Further, a number of significant findings related to nsNSAIDs and selective COX-2 inhibitors have been described here, but these results cannot be considered to represent the effects of other NSAIDs. The NSAIDs are a complex class of drugs that are often dose-dependent and act along a spectrum on multiple pathways.

Conclusion

In this study we attempted to describe the effects of non-selective COX and selective COX-2 inhibitors on the normally perfused myocardium. Celecoxib caused increased blood pressure and protein peroxidation, while naproxen caused decreased tissue prostacyclin and increased thromboxane, as well as decreased numbers of apoptotic cells. Both drugs altered the microvascular response to serotonin, reduced serum prostaglandin levels, and decreased arteriogenesis. Importantly, neither drug lead to an alteration of baseline myocardial perfusion or perfusion during rapid pacing. Overall, both non-selective and selective COX inhibition result in altered systemic and myocardial physiology. The impact of these drugs appears to be quite complex, and may have implications regarding cardiovascular risk.

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Abbreviations

ADP	adenosine diphosphate
AAR	myocardial area at risk
COX	cyclooxygenase
FGF	fibroblast growth factor
LAD	left anterior descending coronary artery

LCx	left circumflex coronary artery
nsNSAID	non-selective non-steroidal anti-inflammatory drug
PGI₂	prostacyclin
RPP	rate pressure product
SMA	smooth muscle actin
SNP	sodium nitroprusside
TXA₂	thromboxane
VEGF	vascular endothelial growth factor

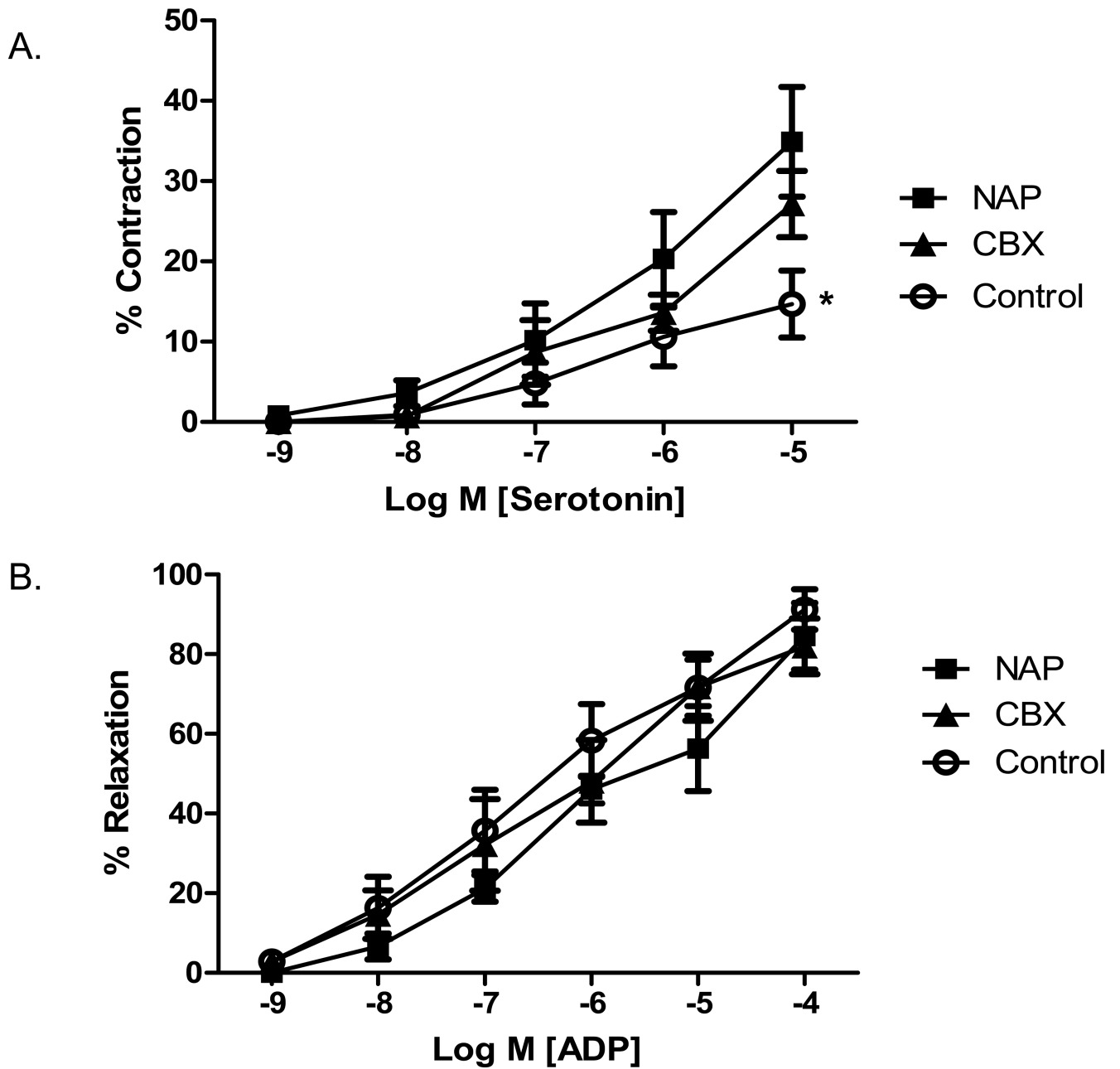


Figure 1. Microvessel reactivity. A) Microvessels harvested from the NAP and CBX group demonstrated increased contraction responses to serotonin as compared to the control (* $p=0.003$ for control v. NAP and CBX). B) There was no difference in the microvascular responses to ADP, an endothelial dependent vasorelaxation agent.

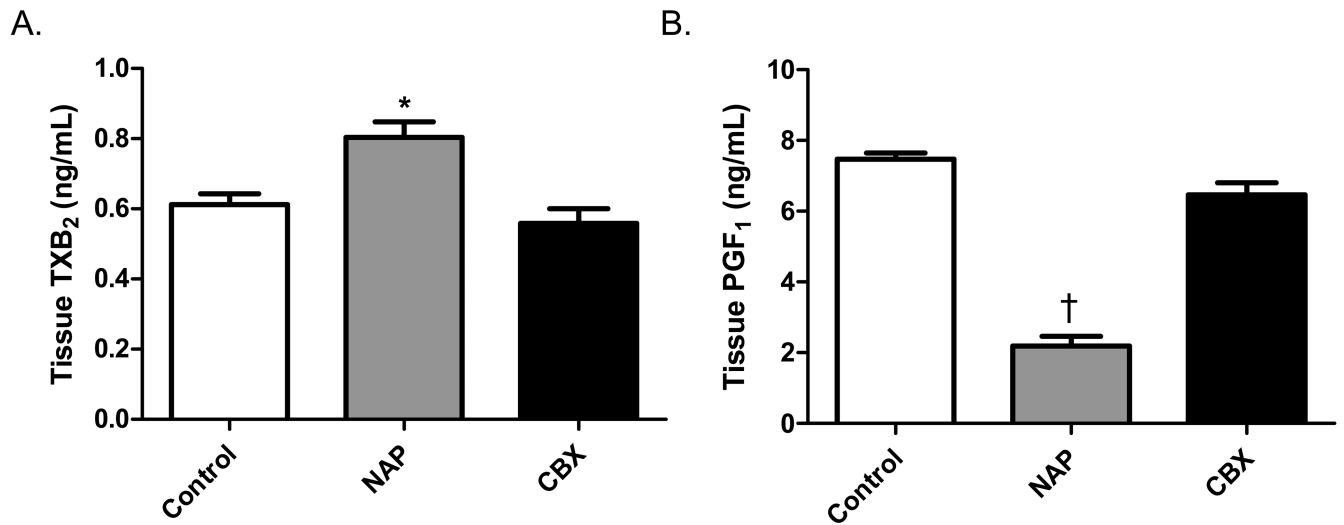


Figure 2. Prostaglandin levels. A) Animals in the NAP group demonstrated increased levels of thromboxane A₂ (TXA₂) as compared to the control and CBX groups (* p= 0.003). The NAP group was found to have lower levels of prostacyclin I₂ (PGI₂) as compared to the control and CBX groups († p< 0.001). There was no difference between the control and CBX groups in TXA₂ or PGI₂ levels (p> 0.05 for both).

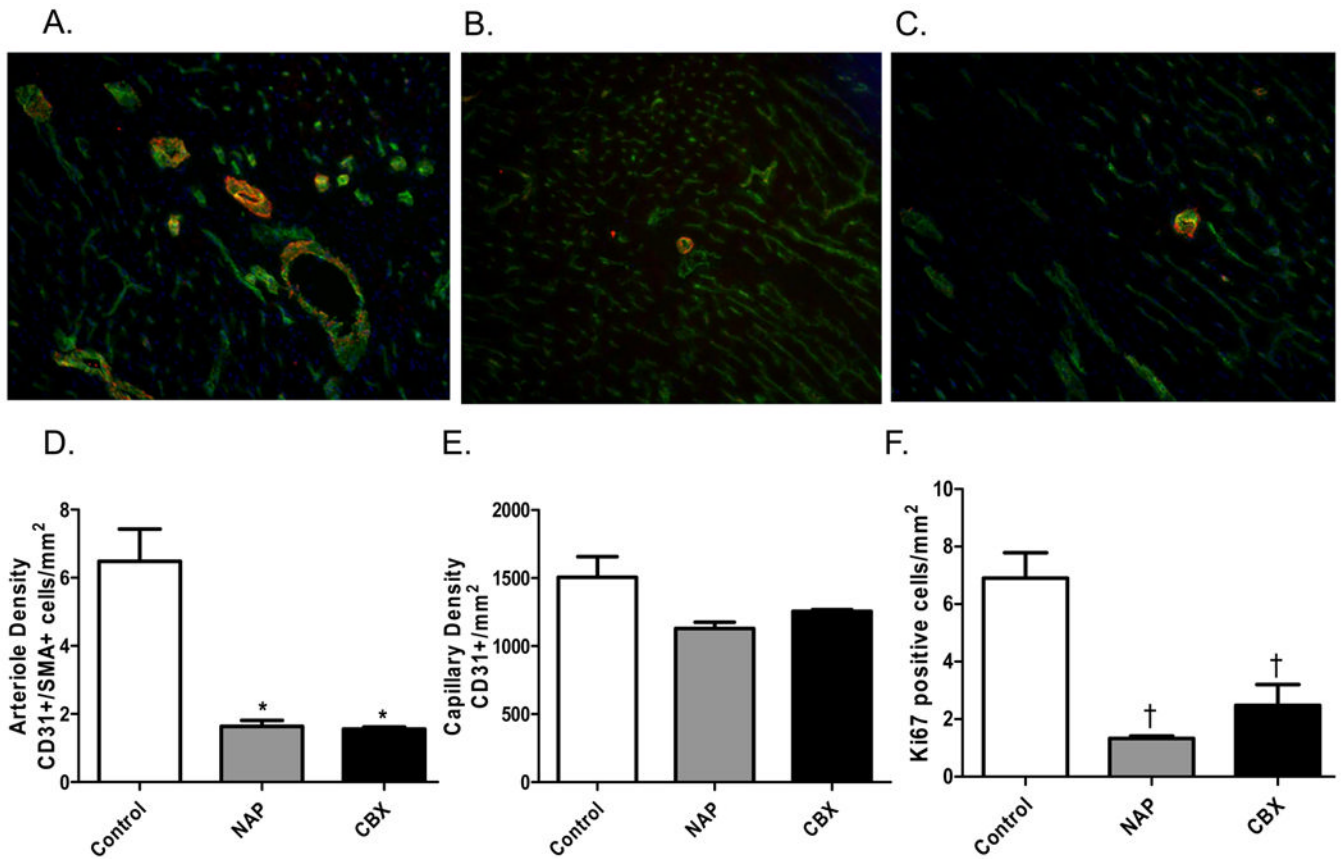


Figure 3.

Arteriogenesis and Angiogenesis. The control group demonstrated increased arteriole density by co-staining for CD-31 (an endothelial marker- green), smooth muscle actin (SMA- red), and DAPI (nuclear stain- blue) as compared to the NAP and CBX groups. A) Control group. B) NAP group. C) CBX group. All images are at 160 \times . D) Quantification of arteriolar density in the myocardium ($p < 0.001$ for control v. NAP and CBX). E) There was a trend toward decreased capillary density by CD-31 staining in the NAP and CBX groups ($p = 0.12$). F) Endothelial proliferation, assessed by co-staining with Ki-67 (proliferation marker) and CD-31, was significantly decreased in the NAP and CBX groups as compared to the control ($p < 0.001$).

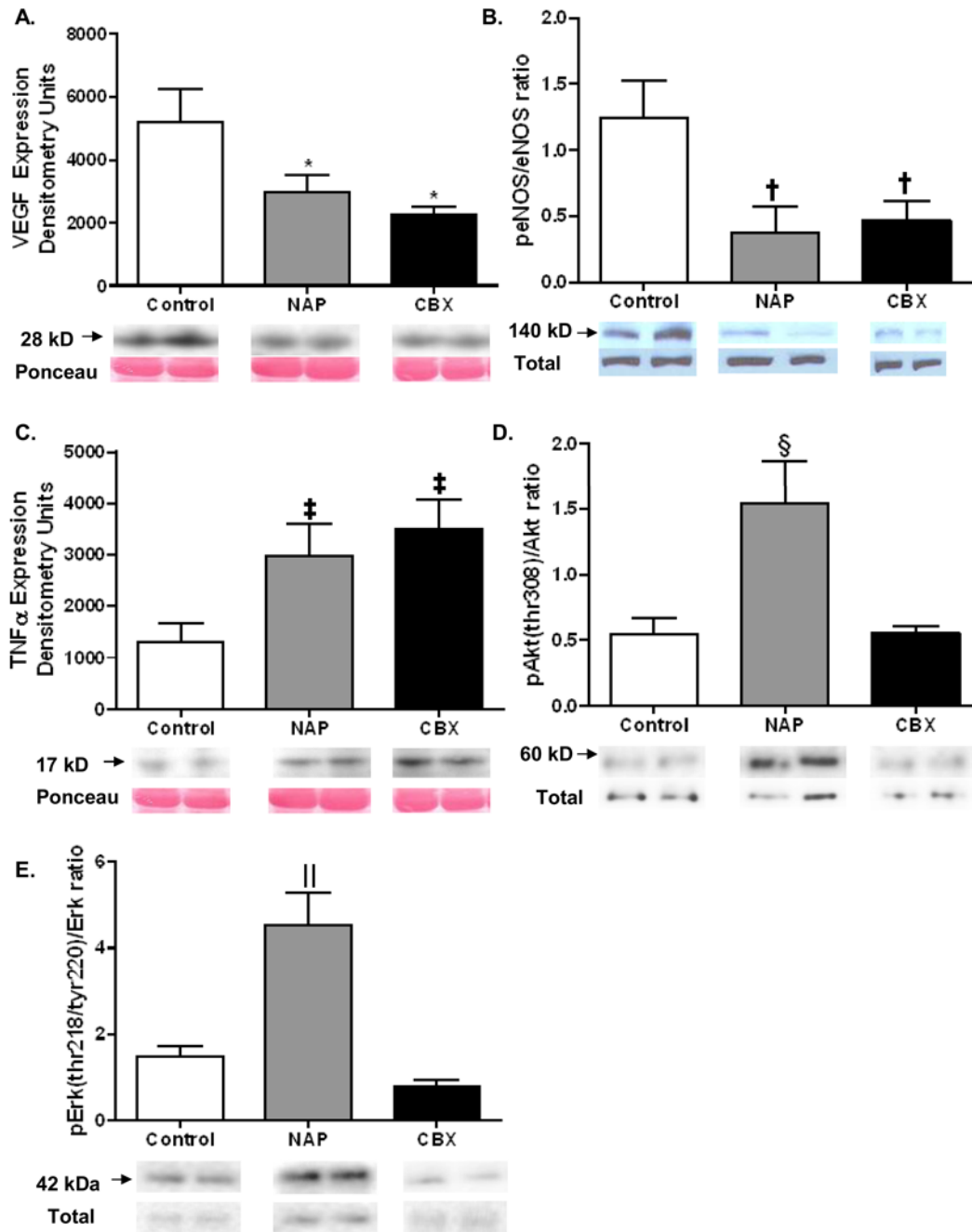


Figure 4.

Immunoblotting. A) Expression of the angiogenic protein VEGF was highest in the control group, and significantly decreased in both the NAP and CBX groups (* $p=0.03$). B) The expression of the vasodilatory, pro-angiogenic protein phospho-eNOS was highest in the control group, and significantly downregulated in the drug treatment groups († $p=0.03$). C) Myocardial TNF α levels were upregulated in the NAP and CBX groups (‡ $p=0.02$). D and E) Levels of activated Akt and ERK, normalized to total levels, were increased in the NAP group (§ $p=0.004$ and || <0.001 , respectively).

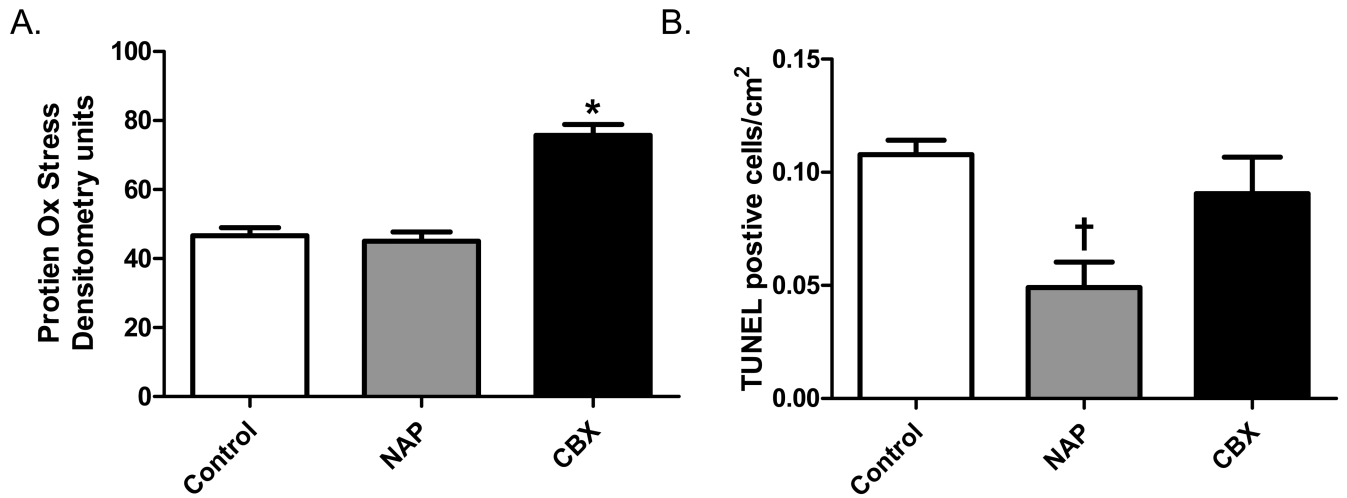


Figure 5. Levels of Oxidative stress and Apoptosis. Protein peroxidation was increased in the CBX group as compared to the control and NAP groups (* $p < 0.001$). Apoptotic cell death was decreased in the NAP groups vs. control and CBX groups († $p = 0.007$).

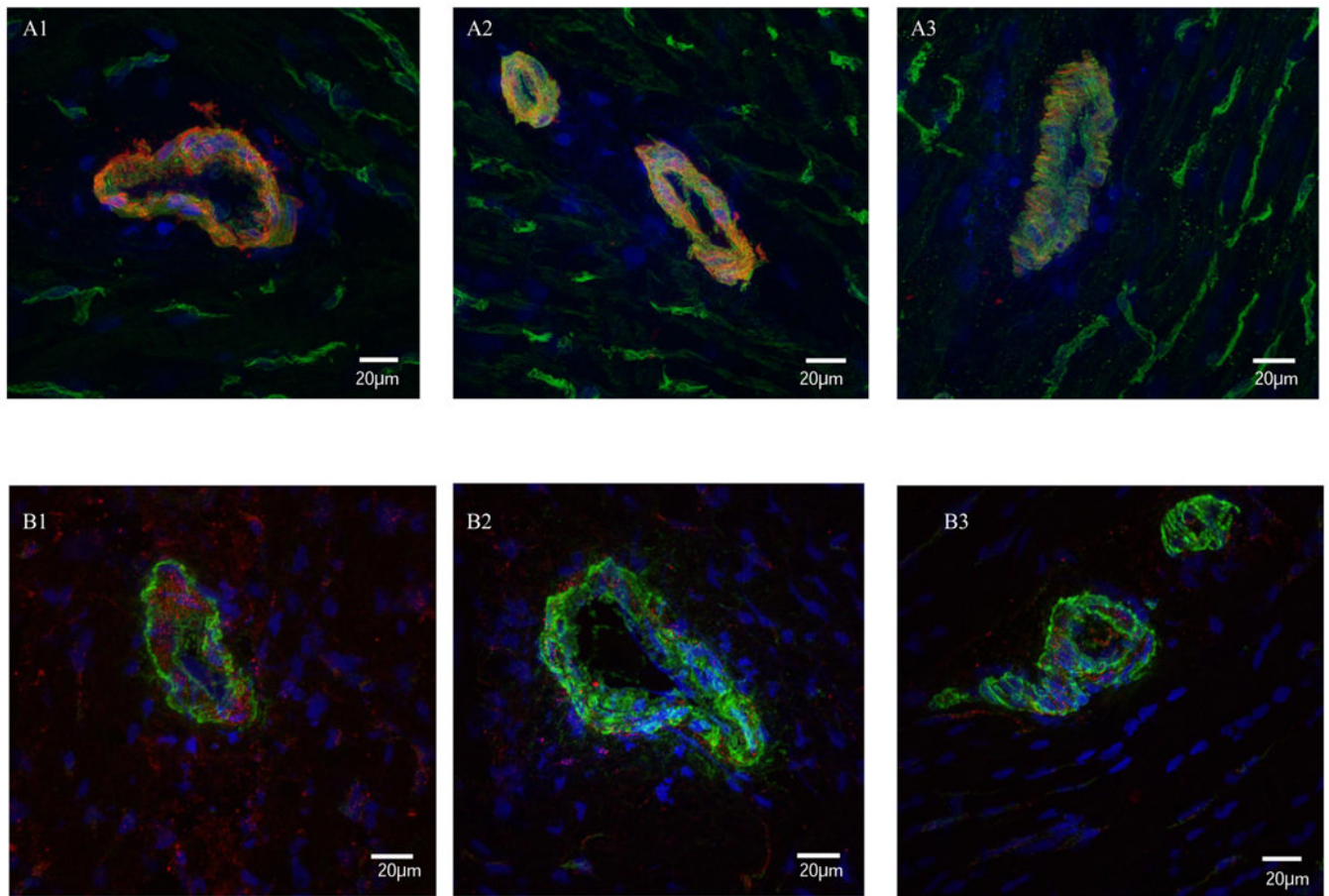


Figure 6. Immunostaining for thromboxane synthase (TS) and receptor (TR). Co-staining for TS and SMA (A) demonstrated localization in the vascular smooth muscle, and no obvious differences between groups; 1) control, 2) NAP, and 3) CBX. Immunostaining for TR and SMA (B) also showed localization in the vascular smooth muscle and no major differences in expression levels between groups; 1) control, 2) NAP, and 3) CBX.

Table 1

Physiologic measurements. SBP- systolic blood pressure, RPP- rate pressure product.

	Control	NAP	CBX	p-value
SBP (mmHg)	69 ± 6.6	53 ± 5.4	96 ± 8.5	0.01
RPP (mmHg*beats/min)	9012 ± 1302	5665 ± 599	9029 ± 879	0.04
Blood Flow- Rest (mL/min/gram)	0.86 ± 0.17	1.22 ± 0.18	1.0 ± 0.18	0.36
Blood Flow- Pacing (mL/min/gram)	0.96 ± 0.13	0.86 ± 0.06	0.86 ± 0.18	0.84