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High-fat Diet Alters Prostanoid Balance and Perfusion in Ischemic Myocardium of Naproxen-treated Swine

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Abstract

Background—The effect of non-steroidal anti-inflammatory drugs (NSAIDs) on the cardiovascular system remains controversial, especially in patients with cardiovascular comorbidities. We used a swine model of chronic myocardial ischemia to investigate whether hypercholesterolemia alters the cardiovascular effects of the non-selective NSAID naproxen.

Methods—Yorkshire swine were fed normal chow (NAP, n=7) or high-fat diet (HF-NAP, n=8). Chronic myocardial ischemia was created in all animals by left circumflex ameroid constrictor placement. All swine were started on oral naproxen (440 mg/day) at the time of ameroid placement. After 7 weeks, myocardial perfusion and microvessel reactivity in the ischemic territory were assessed. Tissue levels of prostanoid metabolites 11-dehydrothromboxane B2 (11-d-TXB₂) and 6-keto-prostaglandin F1- α (6-k-PGF_{1 α}) were measured. Tissue was analyzed for capillary density and protein expression.

Results—Myocardial perfusion was significantly decreased in the HF-NAP group both at rest and during ventricular pacing. Microvessel relaxation responses to sodium nitroprusside and adenosine 5'-diphosphate were similar between groups. Tissue 11-d-TXB₂ levels were similar between groups, but tissue 6-k-PGF_{1 α} was significantly decreased in the HF-NAP group (p=0.001). Expression of thromboxane synthase was significantly higher in the HF-NAP group (p=0.02), while prostacyclin synthase expression was significantly decreased in the HF-NAP group (p=0.04). Capillary density was higher in the HF-NAP group (p=0.005). Pro-angiogenic VEGF (p=0.0002) and Akt (p=0.01) were downregulated in the HF-NAP group.

Conclusions—A high-fat diet impairs tissue perfusion in ischemic myocardium of naproxen-treated swine by shifting the prostanoid balance to favor production of thromboxane over

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DISCLOSURES

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prostacyclin. Thus dietary modification may improve myocardial blood flow and alter the safety profile in chronically ischemic cardiac patients taking naproxen.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medications worldwide for the treatment of postoperative pain, inflammatory conditions, and fever. They inhibit the conversion of arachadonic acid into prostaglandin H₂ by cyclooxygenase (COX), decreasing the downstream synthesis of prostaglandins E₂, D₂, and F₂, which are responsible for the symptom that NSAIDs are commonly used to treat. However, prostaglandin H₂ is also metabolized into prostacyclin and thromboxane A₂, two potent vasoactive prostanoids with directly opposing effects. Prostacyclin, primarily produced by endothelial cells, is a vasodilator and inhibits platelet aggregation, while thromboxane-A₂, produced primarily by platelets, is a vasoconstrictor and promotes platelet aggregation.

Recent studies shed light on the cardiovascular risk of NSAIDs, in particular COX-2-specific inhibitors such as rofecoxib (Vioxx)(1). These drugs were thought to increase the risk of myocardial infarction and other thrombotic events through specific inhibition of prostacyclin, shifting prostanoid balance to favor thromboxane A₂(2). Predictably, attention then turned towards traditional nonselective COX inhibitors such as naproxen, ibuprofen, and diclofenac, as they inhibit both COX-1 and COX-2. Numerous groups reported that non-selective NSAIDs also increase cardiac risk significantly(3– 5). In fact, all NSAIDs (with the exception of aspirin) carry a warning from the United States Food and Drug Administration because of their perceived cardiovascular risk, and are contraindicated immediately following cardiac bypass surgery(6). Naproxen is perhaps the most controversial of these traditional NSAIDs. Although generally considered to be the safest of the group, it has been shown in different observational studies to have protective(7), insignificant (8), or deleterious effects(9) with regard to cardiovascular risk.

The disparity in findings of these studies may stem partially from variation in patient characteristics. Most of these studies enrolled patients with conditions such as rheumatoid arthritis and Alzheimer's disease, and do not account for comorbidities such as hypercholesterolemia and chronic ischemia, which are high in these elderly populations. These conditions lead to endothelial dysfunction and increased oxidative stress that may alter the effect of NSAIDs on myocardial angiogenesis, vessel function, and perfusion. These patients may also take aspirin or other cardiovascular medications that further confound results. In this study, we used a swine model of chronic ischemia to investigate the effects of hypercholesterolemia on the cardiovascular response to the NSAID naproxen.

METHODS

Study Design

Yorkshire miniswine (Parsons Research, Amherst, MA) were divided into two groups. One was fed normal chow for the duration of the experiment (NAP, n=7), and the other was started on a high cholesterol diet consisting of 4% cholesterol, 17.2% coconut oil, 2.3% corn oil, 1.5% sodium cholate, and 75% regular chow (Sinclair Research, Columbia, MO) at 4 weeks of age (HF-NAP, n=8). At 8 weeks of age, animals underwent ameroid constrictor placement on the proximal left circumflex coronary artery (LCx, Research Instruments SW, Escondido, CA). For all surgical procedures, anesthesia was induced with intramuscular telazol (4.4 mg/kg) 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 12–20 breaths/min. During the first procedure, the pericardium was opened through a left mini-thoracotomy,

and a titanium ameroid constrictor (1.75–2.25 mm internal diameter) was placed around the proximal LCx. All animals were then started on oral naproxen (440 mg once daily).

Seven weeks after ameroid placement, swine were again anesthetized, the heart was exposed and physiologic measurements were taken, followed by blood draw for serum lipid measurements and cardiac harvest. Myocardial samples were rapidly frozen in liquid nitrogen (molecular studies and immunohistochemistry) or placed in 4 °C Krebs solution (microvessel reactivity studies).

All experiments were approved by the Beth Israel Deaconess Medical Center and Rhode Island Hospital Institutional Animal Care and Use Committees. Animals were cared for 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

Heart rate (HR), mean arterial pressure (MAP), developed left ventricular pressure (DLVP), first derivative of LV pressure (+dP/dt), and regional LV function in the LCx territory were recorded prior to cardiac harvest using single-sensor pressure catheters (Millar Instruments, Houston, TX) and the Sonometrics system (Sonometrics Corp. London, ON, Canada) as previously described(10).

Myocardial Perfusion Analysis

Myocardial perfusion was measured via isotope-labeled microspheres (BioPAL, Worcester, MA) as previously described(11). Briefly, 1.5×10^7 gold-labeled microspheres were injected during temporary LCx occlusion at the first surgical procedure to identify the ischemic area at risk (AAR). Lutetium (resting heart rate) and Europium (pacing to 150 beats/minute) labeled microspheres were injected at the final procedure while simultaneously withdrawing arterial blood from a femoral artery catheter. LV samples were dried in a 60° C oven for at least 48 hours, then exposed to neutron beams and microsphere densities measured using a gamma counter (BioPAL). Myocardial blood flow in the AAR was determined using the following equation:

$$\text{Blood flow} = (\text{withdrawal rate} / \text{tissue weight}) \times (\text{tissue microsphere count} / \text{blood microsphere count})$$

Microvessel Studies

Coronary arterioles (80–180µm diameter) from the AAR were isolated and placed in a microvessel chamber as described previously(11). Vessels were precontracted with thromboxane-A₂ analog U46619 (0.1–1.0 µM), then treated with endothelium-independent vasodilator sodium nitroprusside (SNP, 10^{-9} to 10^{-4} mol/L) and endothelium-dependent vasodilator adenosine 5'-diphosphate (ADP, 10^{-9} to 10^{-4} mol/L). Responses were defined as percent relaxation of the precontracted diameter. All reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Tissue Prostaglandin Assays

Tissue levels of the stable breakdown products of thromboxane-A₂ and prostacyclin, 11-dehydrothromboxane-B₂ (11-d-TXB₂) and 6-keto prostaglandin-F_{1α} (6-k-PGF_{1α}), respectively, were measured using ELISA kits according to the manufacturer's specifications (Neogen Corp, Lexington, KY). Briefly, tissue lysates underwent liquid-liquid exchange, extraction, and concentration, and were then loaded into 96-well plates containing

antibody to either 11-d-TXB₂ or 6-k-PGF_{1α}. Plates were washed and colorimetric substrate added. Absorbance was read at 650 nm, and results plotted against a standard curve.

Immunoblotting

Sixty micrograms of total protein from AAR homogenates were fractionated by SDS-PAGE (Invitrogen, San Diego, CA) and transferred to PVDF membranes (Millipore, Bedford, MA). Membranes were incubated with antibodies against thromboxane synthase (Abcam, San Francisco, CA), prostacyclin synthase (Cayman Chemical, Ann Arbor, MI), vascular endothelial growth factor (VEGF), and pan-Akt (Cell Signaling Technology, Danvers, MA) at dilutions recommended by the manufacturer, followed by the appropriate HRP-linked secondary antibodies (1:3000, Jackson ImmunoResearch, West Grove, PA). Immune complexes were visualized via chemiluminescence (Amersham, Piscataway, NJ) and photographed using GeneSnap software (Syngene, Cambridge, England). Densitometry was performed using Image J software (NIH, Bethesda, MD). Ponceau staining was used to ensure equal protein loading.

Immunohistochemical Staining for Capillary density

12 μm-thick frozen sections of myocardium were formalin fixed, then incubated with antibody against CD-31 (1:200, R&D Systems, Minneapolis, MN), followed by secondary DyLight-conjugated antigoat antibody (1:100, Jackson ImmunoResearch, West Grove, PA). Sections were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Photomicrographs were taken with a Zeiss Axiolab microscope (Carl Zeiss Inc, Thornwood, NY) under 20x magnification. Capillaries, defined as CD31 positive structures between 5–25 μm² in cross-sectional area, were counted using Image J software (National Institute of Health, Bethesda, MD). Data is presented as capillaries/mm².

Statistical Analysis

All results are presented as mean ± SEM. Microvessel responses were analyzed using two-way, repeated-measures ANOVA with a post-hoc Bonferroni test. All other comparisons were carried out using an unpaired, two-tailed t-test using GraphPad Prism 5.0 Software (GraphPad Software Inc., San Diego, CA).

RESULTS

Hypercholesterolemic swine model

Both groups started with 8 animals. One animal from the NAP group died from irreversible ventricular fibrillation during the terminal surgery, for a final n=7 in the NAP group. All animals from the HF-NAP group survived the entire experiment for a final n=8. Body weight was not significantly different between the two groups, though HF-NAP animals tended to be heavier (27.7 ± 1.8 kg in NAP vs. 30.7 ± 0.9 kg in HF-NAP, p=0.15). Serum cholesterol was significantly increased in the HF-NAP group compared to the NAP group (79 ± 5 mg/dL in NAP vs. 479 ± 66 mg/dL in HF-NAP, p = 0.001). Both serum LDL (40.2 ± 3.3 mg/dL in NAP vs. 308.5 ± 54 mg/dL in HF-NAP, p<0.0001) and HDL (28.0 ± 2.2 mg/dL in NAP vs. 150.0 ± 14.7 mg/dL in HF-NAP, p<0.0001) were significantly increased in the HF-NAP group, as was the ratio of LDL:HDL (1.46 ± 0.11 in NAP vs 2.13 ± 0.23 in HF-NAP, p=0.03).

Global and regional myocardial function

There was no significant difference in heart rate, mean arterial pressure, developed left ventricular (LV) pressure, or LV contractility (dP/dt) between groups (Figure 1A–D).

Regional myocardial contractility in the AAR as measured by segmental shortening on the vertical and horizontal axes was similar between groups as well (Figure 1E–F).

Myocardial perfusion

Myocardial perfusion in the AAR was significantly lower in the HF-NAP group both at rest and under ventricular pacing conditions (Figure 2A–B).

Microvessel function

Microvessel relaxation responses to the endothelium-dependent ADP and endothelium-independent SNP were not significantly different between groups (Figure 3A–B).

Tissue prostaglandin assays

Tissue levels of 11-d-TXB₂ in the AAR were similar between groups, while 6-k-PGF_{1α} levels were significantly lower in the HF-NAP group compared to the NAP group (Figure 4A–B).

Immunoblotting

Expression of thromboxane synthase was significantly higher in the HF-NAP group, while prostacyclin synthase expression was lower in the HF-NAP group. Akt and VEGF expression were significantly lower in the HF-NAP group as well (Figure 5A–D).

Capillary density

Capillary density as measured by immunohistochemical staining was significantly higher in the HF-NAP group than the NAP group (148 ± 15 vessels/mm² in NAP vs. 200 ± 8 vessels/mm² in HF-NAP, $p=0.005$).

DISCUSSION

Naproxen is a widely used drug for the treatment of pain and inflammatory conditions that is available in the United States without prescription. Its gastrointestinal side-effects have been well documented, but recent investigation into its cardiovascular safety has been inconclusive. The majority of evidence favors either a negligible or slightly protective effect of the drug on cardiovascular risk(8), but its specific effects on the heart remain unclear. Furthermore, the effect of cardiovascular comorbidities such as chronic ischemia and hypercholesterolemia on the safety profile of naproxen is unknown. We have previously completed two studies that examine the specific effects of naproxen on chronically ischemic myocardium in comparison to non-drug treated controls. We found that, in normal diet swine, naproxen treatment increased myocardial blood flow, but also increased microvessel contraction responses to serotonin and endothelin-1, decreased tissue prostacyclin levels only, and seemed to inhibit angiogenesis and decrease VEGF and VEGFR₂ expression (12). In contrast, in hypercholesterolemic swine, naproxen treatment had no effect on blood flow, but improved endothelium-dependent vasodilation, while inhibiting production of both prostacyclin and thromboxane (Chu et al, submitted, Am J Phys, 2011). Thus hypercholesterolemia clearly alters the effect of COX inhibition with naproxen. We designed this study in order to better elucidate the effects of hypercholesterolemia on COX inhibition. We showed that hypercholesterolemia alters prostanoid balance and reduces perfusion in the ischemic territory of naproxen-treated swine. Myocardial perfusion is a predictor of angina frequency and quality of life in patients with chronic ischemia. Furthermore, decreased prostacyclin levels may predispose to thrombogenesis and

atherosclerosis(13). Thus, these findings have important implications for cardiac patients taking NSAIDs.

In 1999, FitzGerald *et al* hypothesized that COX-2 inhibition leads to selective inhibition of prostacyclin synthesis, thereby allowing the vasoconstrictive and pro-thrombotic effects of thromboxane A₂ to predominate and predisposing to cardiovascular morbidity(2). We previously found that, in normocholesterolemic pigs, naproxen selectively decreased prostacyclin levels in ischemic myocardium compared to untreated controls, while tissue thromboxane levels were actually increased(12). Unexpectedly, myocardial perfusion in these animals was significantly increased compared to untreated animals, contradicting the so-called FitzGerald hypothesis. However, FitzGerald himself and others acknowledged that thromboxane-prostacyclin balance is only one facet of vascular regulation(14, 15). In this study, we found that both prostacyclin-to-thromboxane ratio and myocardial perfusion were decreased in the ischemic territory of hypercholesterolemic swine, more consistent with the FitzGerald hypothesis. Thus, conditions such as hypercholesterolemia that affect the cardiovascular environment may play a significant role in the biologic effects of prostanoids.

Through a variety of mechanisms, hypercholesterolemia leads to endothelial dysfunction and decreased angiogenesis in ischemic myocardium as evidenced by increased contractile and worsened relaxation responses of coronary microvessels and decreased capillary density(16, 17). In this study, though the hypercholesterolemic group did demonstrate a decrease in angiogenic proteins Akt and VEGF, there was no difference in microvessel function, and capillary density was actually increased. Some studies have shown that COX inhibition counteracts the endothelial dysfunction brought on by dyslipidemia and sepsis(18, 19). However, we found that the alterations in prostanoid balance induced by hypercholesterolemia outweigh any benefits of COX inhibition with regard to endothelial dysfunction, leading to an overall decrease in myocardial perfusion. The increase in capillary density may be an attempt to compensate for this reduced-flow state.

Somewhat surprisingly, global ventricular function and regional contractility were not decreased in the HCX group, despite significantly impaired perfusion. There are several possible explanations for this. The preservation of global and regional myocardial function could partially be due to different substrate utilization. We found in a recent study that hypercholesterolemic swine tended to have increased MAP, dLVP, and had significantly increased LV contractility compared to normal diet swine in a model of acute ischemia-reperfusion(20). We hypothesized that this may be due to an increase in circulating short-chain fatty acids, which may be a preferred energy source in diseased myocardium(20, 21). Furthermore, it has been shown that the activity of non-ischemic myocardium increases to compensate for local ischemia, leaving overall heart function unaffected(22). It is surprising, however, that we did not see a decrease in contractility in the ischemic territory. One possibility is that both normal and hypercholesterolemic swine exhibit similar degrees of myocardial hibernation in response to chronic ischemia, so that even though perfusion is higher in the normal diet group, contractility is equally impaired. Additionally, it is possible that the sonometric probes used to measure regional function were not placed entirely within the ischemic territory in some animals, and differences in the depth of insertion of the probes can affect the measurements as well. We believe that the perfusion measurements are more accurate than the functional measurements, as they are calculated from tissue that is known to be ischemic.

Limitations

Several limitations to this study should be mentioned. Firstly, we examined the effects of cholesterol and naproxen on the vascular effects of prostanoids, but did not examine platelet function. Increased thromboxane-to-prostacyclin ratio in the hypercholesterolemic group

may also lead to increased thrombogenesis, further increasing cardiac risk. Secondly, though we chose a swine model to most closely approximate the human cardiovascular system, it is possible that the response to NSAIDs and production of prostanoids in swine differs from that of humans. Finally, because the prostanoids themselves are so unstable, we were forced to quantify them indirectly by measuring their stable metabolites. Though these methods have been previously validated(23, 24), they likely do not completely represent the *in vitro* circulating levels of prostacyclin and thromboxane. We attempted to limit this potential source of variability by measuring the metabolites directly from the tissue of interest.

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Figure 1. Global and regional cardiac function. Shown are measurements of heart rate (HR, A), mean arterial pressure (MAP, B), developed left ventricular pressure (DLVP, C), contractility measured as first derivative of LV pressure (dP/dt, D), and regional contractility in the AAR as measured by segmental shortening on the vertical (VSS, E) and horizontal (HSS, F) axes at the time of cardiac harvest. There was no significant difference in any of these measures between the NAP and HFNAP groups.

Figure 2. Myocardial perfusion. Myocardial perfusion in the AAR was measured by radiolabeled microspheres at resting heart rate and under ventricular pacing. Both rest flow (A) and paced flow (B) were significantly decreased in the HF-NAP group compared to the NAP group.

Figure 3. Microvessel function. Microvessels from the AAR were treated with endothelium-dependent vasodilator ADP (A) and endothelium-independent vasodilator SNP (B). There was no significant difference between groups in the relaxation response to either drug at any drug concentration.

Figure 4.

Tissue prostanoid levels. Levels of 11-d-TXB₂ and 6-k-PGF_{1α}, the direct metabolites of thromboxane and prostacyclin, respectively, were measured in the AAR. There was no significant difference in tissue 11-d-TXB₂ between groups (A), while tissue 6-k-PGF_{1α} was significantly lower in the HF-NAP group (B).

Figure 5. Immunoblotting results. Shown are representative bands and expression of thromboxane synthase (TS), prostacyclin synthase (PGIS), Akt, and VEGF. Thromboxane synthase was significantly increased in the AAR of HF-NAP animals (A), while prostacyclin synthase was decreased in HF-NAP (B). Both Akt (C) and VEGF (D) were significantly decreased in the HF-NAP group.