High-fat diet alters prostanoid balance and perfusion in ischemic myocardium of naproxen-treated swine

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Background. *The effect of nonsteroidal 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 nonselective NSAID naproxen.*
Methods. *Yorkshire swine were fed normal chow (NAP;* n = *7) or a 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-TXB_2) and 6-keto-prostaglandin F1-α (6-k-$PGF_{1\alpha}$) 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-TXB_2 levels were similar between groups, but tissue 6-k-$PGF_{1\alpha}$ was significantly decreased in the HF-NAP group (*P = *.001). Expression of thromboxane synthase was significantly higher in the HF-NAP group (*P = *.02), while prostacyclin synthase expression was significantly decreased in the HF-NAP group (*P = *.04). Capillary density was higher in the HF-NAP group (*P = *.005). Proangiogenic vascular endothelial growth factor (VEGF;* P = *.0002) and Akt (*P = *.01) were downregulated in the HF-NAP group.*
Conclusion. *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 prostacyclin. Dietary modification may improve myocardial blood flow and alter the safety profile in chronically ischemic cardiac patients taking naproxen. (Surgery 2011;150:490-6.)*

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Nonsteroidal 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 H_2 by cyclooxygenase (COX), decreasing the downstream synthesis of prostaglandins E_2, D_2, and F_2, which are responsible for the symptom that NSAIDs are commonly used to treat. However, prostaglandin H_2 is also metabolized into prostacyclin and thromboxane A_2, 2 potent vasoactive prostanoids with directly opposing effects. Prostacyclin, primarily produced by endothelial cells, is a vasodilator and inhibits platelet aggregation, while thromboxane-A_2, 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.[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 A_2.[2] Predictably, attention then turned toward traditional nonselective COX inhibitors, such as naproxen, ibuprofen, and diclofenac, because they inhibit both COX-1 and COX-2. Numerous groups reported that nonselective NSAIDs also increase cardiac risk significantly.[3-5] In fact, all NSAIDs (with the exception of aspirin) carry a warning from the US Food and Drug Administration because of their perceived cardiovascular risk, and are contraindicated immediately after 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 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 2 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 to 2 L/minute and 3.0% isoflurane. The animals were intubated and mechanically ventilated at 12 to 20 breaths per minute. During the first procedure, the pericardium was opened through a left minithoracotomy, 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 "Priciples of Laboratory Animal Care" are recommendations promulgated by the National Society for Medical Research that have been incorprated into the NIH Guide for the Care and Use of Laboratory Animals. (National Institutes of Health 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 before 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/min) 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:

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

Microvessel studies. Coronary arterioles (80–180 μm in diameter) from the AAR were isolated and placed in a microvessel chamber as described previously.[11] Vessels were preconstricted with thromboxane-A_2 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 preconstricted diameter. All reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Tissue prostaglandin assays. Tissue levels of the stable breakdown products of thromboxane-A_2 and prostacyclin, 11-dehydrothromboxane-B_2 (11-d-TXB_2), and 6-keto prostaglandin-$F_{1\alpha}$ (6-k-$PGF_{1\alpha}$), respectively, were measured using enzyme-linked immunosorbent assay 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-TXB_2 or 6-k-$PGF_{1\alpha}$. Plates were washed and colorimetric substrate added. Absorbance was read at 650 nm, and the results were plotted against a standard curve.

Immunoblotting. Sixty micrograms of total protein from AAR homogenates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Invitrogen, San Diego, CA) and transferred to polyvinylidene fluoride 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 manufacturers, followed by the appropriate horseradish peroxidase–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 (National Institutes of Health, Bethesda, MD). Ponceau staining was used to ensure equal protein loading.

Immunohistochemical staining for capillary density. Twelve micrometer thick frozen sections of myocardium were fixed in formalin and incubated with antibody against CD31 (1:200; R&D Systems, Minneapolis, MN), followed by secondary DyLight-conjugated anti-goat antibody (1:100; Jackson ImmunoResearch). Sections were mounted in Vectashield with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Photomicrographs were taken with a Zeiss Axiolab microscope (Carl Zeiss Inc, Thornwood, NY) under 20× magnification. Capillaries, defined as CD31-positive structures between 5 and 25 μm^2 in a cross-sectional area, were counted using Image J software (National Institutes of Health). Data are presented as capillaries per mm^2.

Statistical analysis. All results are presented as mean ± SEM. Microvessel responses were analyzed using 2-way repeated-measures analysis of variance with a post-hoc Bonferroni test. All other comparisons were carried out using an unpaired, 2-tailed *t* test using GraphPad Prism software (version 5.0; 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 number of 7 animals in the NAP group. All animals from the HF-NAP group survived the entire experiment for a final number of 8 animals. Body weight was not significantly different between the 2 groups, although HF-NAP animals tended to be heavier (27.7 ± 1.8 kg in the NAP group versus 30.7 ± 0.9 kg in the HF-NAP group; $P = .15$). Serum cholesterol was significantly increased in the HF-NAP group compared to the NAP group (79 ± 5 mg/dL in the NAP group versus 479 ± 66 mg/dL in the HF-NAP group; $P = .001$). Both serum low-density lipoprotein cholesterol (40.2 ± 3.3 mg/dL in the NAP group versus 308.5 ± 54 mg/dL in the HF-NAP group; $P < .0001$) and high-density lipoprotein cholesterol (28.0 ± 2.2 mg/dL in the NAP group versus 150.0 ± 14.7 mg/dL in the HF-NAP group; $P < .0001$) were significantly increased in the HF-NAP group, as was the ratio of low- to high-density lipoprotein cholesterol (1.46 ± 0.11 in the NAP group versus 2.13 ± 0.23 in the HF-NAP group; $P = .03$).

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 (Fig 1, *A–D*). Regional myocardial contractility in the AAR as measured by segmental shortening on the vertical and horizontal axes was also similar between groups (Fig 1, *E* and *F*).

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

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

Tissue prostaglandin assays. Tissue levels of 11-d-TXB_2 in the AAR were similar between groups, while

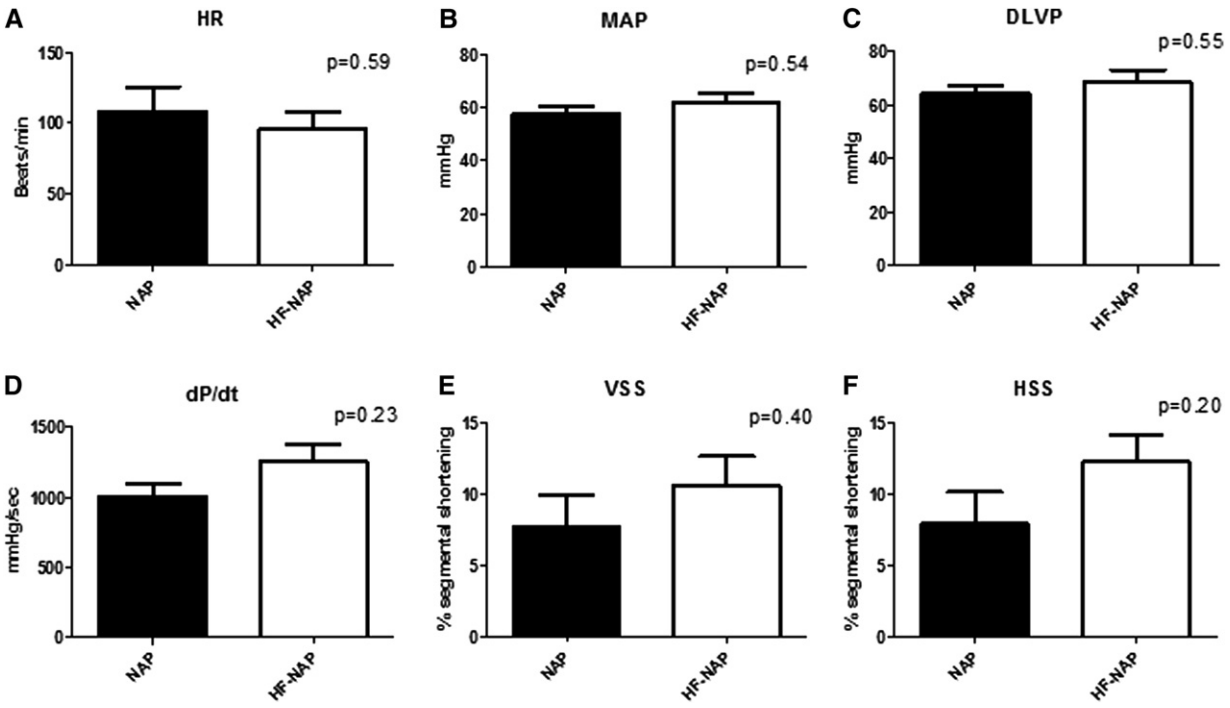

Fig 1. Global and regional cardiac function. Shown are measurements of heart rate (HR, *A*), mean arterial pressure (MAP, *B*), developed left ventricular pressure (DLVP, *C*), contractility measured as first derivative of LV pressure (dP/dt, *D*), and regional contractility in the AAR as measured by 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 normal chow (NAP) and high-cholesterol chow (HF-NAP) groups.

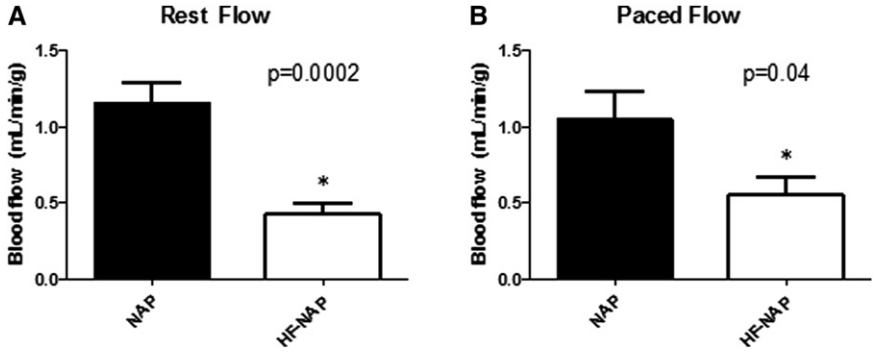

Fig 2. Myocardial perfusion in the area at risk was measured by radiolabeled microspheres at resting heart rate and under ventricular pacing. Both rest flow (*A*) and paced flow (*B*) were significantly decreased in the high-cholesterol chow group compared to the normal chow group.

6-k-$PGF_{1\alpha}$ levels were significantly lower in the HF-NAP group compared to the NAP group (Fig 4, *A* and *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 also significantly lower in the HF-NAP group (Fig 5, *A–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/mm^2 in the NAP group vs 200 ± 8 vessels/mm^2 in the HF-NAP group; $P = .005$).

DISCUSSION

Naproxen is a widely used drug for the treatment of pain and inflammatory conditions, and it 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

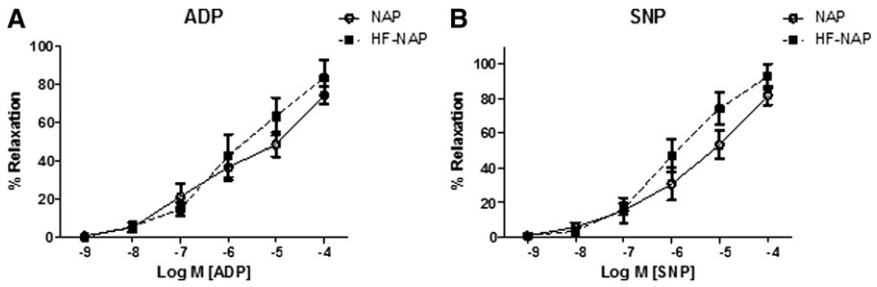

Fig 3. Microvessel function. Microvessels from the area at risk (AAR) were treated with endothelium-dependent vasodilator adenosine diphosphate (*A*) and endothelium-independent vasodilator sodium nitroprusside (*B*). There was no significant difference between groups in the relaxation response to either drug at any drug concentration.

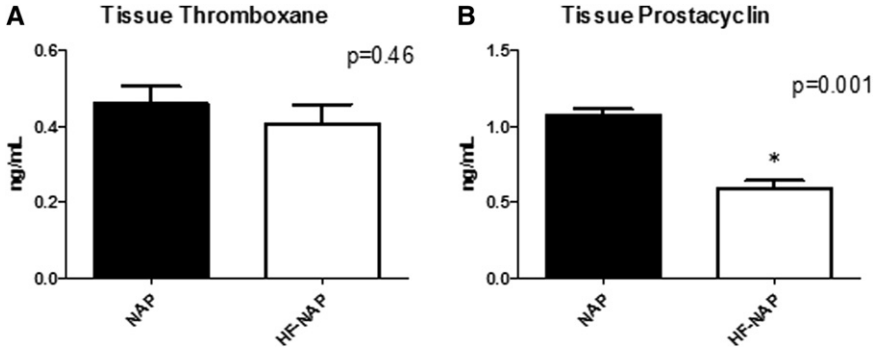

Fig 4. Levels of 11-d-TXB_2 and 6-k-$PGF_{1\alpha}$, the direct metabolites of thromboxane and prostacyclin, respectively, were measured in the area at risk. There was no significant difference in tissue 11-d-TXB_2 between groups (*A*), while tissue 6-k-$PGF_{1\alpha}$ was significantly lower in the high-cholesterol chow (HF-NAP) group (*B*).

protective effect of the drug on cardiovascular risk,[8] but its specific effects on the heart remain unclear. In addition, the effect of cardiovascular comorbidities on the safety profile of naproxen, such as chronic ischemia and hypercholesterolemia, is unknown. We have previously completed 2 studies that examined 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 $VEGFR_2$ 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 (L.M. Chu, MD, unpublished data, 2011). 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 revealed 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. In addition, decreased prostacyclin levels may predispose to thrombogenesis and atherosclerosis.[13] These findings have important implications for cardiac patients taking NSAIDs.

In 1999, McAdam et al[2] hypothesized that COX-2 inhibition leads to selective inhibition of prostacyclin synthesis, thereby allowing the vasoconstrictive and prothrombotic effects of thromboxane A_2 to predominate and predisposing to cardiovascular morbidity. 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

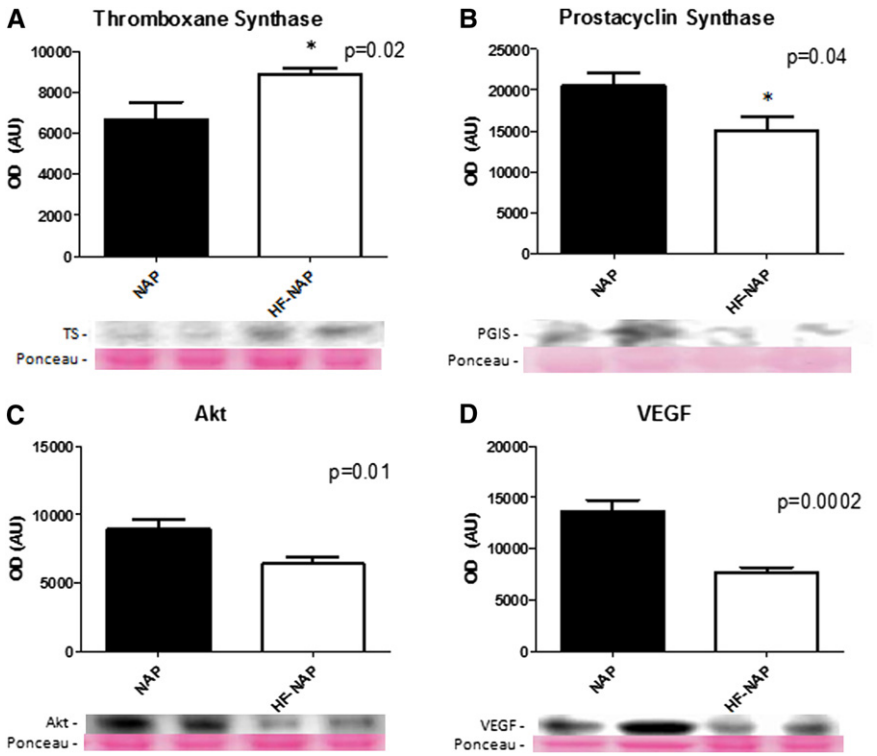

Fig 5. Immunoblotting results. Shown are representative bands and expression of thromboxane synthase (TS), prostacyclin synthase (PGIS), Akt, and vascular endothelial growth factor (VEGF). TS was significantly increased in the area at risk of animals fed high-cholesterol chow (HF-NAP, *A*), while PGIS was decreased in the HF-NAP group (*B*). Both Akt (*C*) and VEGF (*D*) were significantly decreased in the HF-NAP group.

the FitzGerald hypothesis. Conditions such as hypercholesterolemia that affect the cardiovascular environment may therefore 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, although the hypercholesterolemic group did hve 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 HF-NAP group, despite significantly impaired perfusion. There are several possible explanations for this. The preservation of global and regional myocardial function could partially be related to the use of different substrates. We found in a recent study that hypercholesterolemic swine tended to have increased MAP and 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 caused by an increase in circulating short-chain fatty acids, which may be a preferred energy source in diseased myocardium.[20,21] In additon, it has been shown that the activity of nonischemic 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. In addition, 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 also affect the measurements.

We believe that the perfusion measurements are more accurate than the functional measurements, because they are calculated from tissue that is known to be ischemic.

Limitations. Several limitations to this study should be mentioned. First, 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. Second, 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. Although 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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