Beneficial effects of acute inhibition of the oxidative pentose phosphate pathway in the failing heart

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Vimercati C, Qanud K, Mitacchione G, Sosnowska D, Ungvari Z, Sarnari R, Mania D, Patel N, Hintze TH, Gupte SA, Stanley WC, Recchia FA. Beneficial effects of acute inhibition of the oxidative pentose phosphate pathway in the failing heart. Am J Physiol Heart Circ Physiol 306: H709–H717, 2014. First published January 10, 2014; doi:10.1152/ajpheart.00783.2013.—In vitro studies suggested that glucose metabolism through the oxidative pentose phosphate pathway (oxPPP) can paradoxically feed superoxide-generating enzymes in failing hearts. We therefore tested the hypothesis that acute inhibition of the oxPPP reduces oxidative stress and enhances function and metabolism of the failing heart, in vivo. In 10 chronically instrumented dogs, congestive heart failure (HF) was induced by high-frequency cardiac pacing. Myocardial glucose consumption was enhanced by raising arterial glycemia to levels mimicking postprandial peaks, before and after intravenous administration of the oxPPP inhibitor 6-aminonicotinamide (80 mg/kg). Myocardial energy substrate metabolism was measured with radiolabeled glucose and oleic acid, and cardiac 8-isoprostane output was used as an index of oxidative stress. A group of five chronically instrumented, normal dogs served as control. In HF, raising glycemic levels from 80 to ~170 mg/dL increased cardiac isoprostane output by approximately twofold, whereas oxPPP inhibition normalized oxidative stress and enhanced cardiac oxygen consumption, glucose oxidation, and stroke work. In normal hearts glucose infusion did not induce significant changes in cardiac oxidative stress. Myocardial tissue concentration of 6P-glucose 6-phosphate, an intermediate metabolite of the oxPPP, was significantly reduced by ~50% in treated versus nontreated failing hearts, supporting the inhibitory effect of 6-aminonicotinamide. Our study indicates an important contribution of the oxPPP activity to cardiac oxidative stress in HF, which is particularly pronounced during common physiological changes such as postprandial glycemic peaks.

Glucose 6-phosphate dehydrogenase (G6PD) catalyzes the rate-limiting step of the oxidative pentose phosphate pathway (oxPPP) and is the main supplier of cytosolic NADPH (33). The preservation of cellular redox homeostasis, especially in high energy turnover cells such as cardiomyocytes, critically depends on NADPH availability: it has been shown that pharmacological inhibitors of G6PD cause oxidative stress and marked contractile dysfunction in isolated cardiomyocytes (16). Consequently, the G6PD upregulation occurring under various pathological conditions likely serves as a defensive mechanism to protect cells against reactive oxygen species. However, by testing dog and human tissue homogenates, we found that myocardial G6PD upregulation in the failing heart might paradoxically accelerate, rather than prevent, oxidative stress (10, 11). We proposed that the hyperactive oxPPP feeds NADPH-consuming enzymes known as potential superoxide generators in failing hearts, such as NADPH oxidase (13, 24) and uncoupled nitric oxide (NO) synthase (7). If our interpretation is true, G6PD upregulation, aimed at protecting the heart, turns into a detrimental response that contributes to the progression of heart failure (HF). Keeping NADPH production within physiological levels would therefore limit myocardial damage.

We recently tested this hypothesis (12) in mice with a G6PD mutation that causes a 60% reduction in enzyme activity, still compatible with a normal phenotype and mimicking human G6PD deficiency, the most common genetic defect in the world (2, 36). When subjected to myocardial infarction or transverse aortic constriction to induce moderate HF, mutant mice displayed slightly worse, rather than favorable, evolution of left ventricular dysfunction, thus challenging our previous hypothesis. On the other hand, these findings do not support either a critically important protective role of G6PD activity in the diseased heart. However, we could not exclude compensatory mechanisms, very common in mice with inborn genetic defects. For instance, the levels of myocardial NADPH in G6PD mutant mice remained within normal limits. Moreover, in these animals G6PD expression is intrinsically limited, incapable of responding to severe pathological changes. Studies are needed in animals with a normal genome to draw more definitive conclusions regarding the pathological role of G6PD upregulation. Therefore, the aim of the present study was to test the hypothesis that acute pharmacological inhibition of the oxPPP reduces oxidative stress and enhances cardiac function and metabolism in a dog model of decompensated HF. Because the failing heart displays abnormally high glucose consumption (3, 23), we also hypothesized that transient increases in glycemic levels, similar to those found in the postprandial phase, accelerate the flux through the oxPPP and further exacerbate cardiac...
oxidative stress. Our dog model allowed direct blood sampling from aorta and coronary sinus and coronary flow measurements for the quantification of the rate of cardiac glucose, free fatty acids (FFA), and lactate consumption in the conscious state. Transmyocardial blood sampling was also necessary to simultaneously determine changes in cardiac oxidative stress by measuring the output of the plasma marker 8-isoprostane, a stable end-product of oxygen free radical mediated-lipid per-oxidation (22, 25).

MATERIALS AND METHODS

Surgical instrumentation and hemodynamic measurements. Twenty-nine adult, male, mongrel dogs (25–27 kg) were chronically instrumented as previously described (27). Briefly, anesthesia was induced with propofol (6 mg/kg iv) and maintained with 1.5–2% isoflurane during 40% oxygen/60% air ventilation, a thoracotomy was performed in the left fifth intercostal space, a catheter was placed in the descending thoracic aorta, a solid-state pressure gauge (P6.5; Königsberg Instruments) was inserted into the left ventricle through the apex, a Doppler flow transducer (Craig Hartley) was placed around the left circumflex coronary artery, and a pair of pacing leads was fixed on the left ventricular (LV) free wall. Wires and catheters were run subcutaneously to the intrascapular region, the chest was closed in layers, and the pneumothorax was reduced. Antibiotics were given after surgery, and the dogs were allowed to fully recover. After 7–10 days of recovery from surgery, dogs were trained to lie quietly on the laboratory table. The protocol was approved by the Institutional Animal Care and Use Committee of the New York Medical College and conformed to the Guiding Principles for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Experimental protocol. In our experiments we tested the potential role of oxPPP by administering to dogs 80 mg/kg of 6-aminonicotinamide dissolved in 7 ml of DMSO. 6-Aminonicotinamide is a competitive inhibitor of both 6-phosphoglucose dehydrogenase and G6PD (19), the two enzymes of the oxPPP that catalyze the reduction of NADP⁺ to NADPH. The molar weight of this pharmacological agent is 137.1 g/mol, and we have previously found that, in whole tissue homogenates, it is fully effective at the concentration of 5 mM (10, 11). Because there were no data in literature relative to acute administration of 6-aminonicotinamide in dogs and we needed an effect within minutes, we simply estimated, very conservatively, that a bolus of 80 mg/kg, diluted in 2.5–5 ml of circulating blood +1–2 ml of interstitial fluids, would provide tissue concentrations close to 5 mM, sufficient for rapid and sustained oxPPP inhibition. Previous studies in mice found oxPPP inhibition 10 h after administration of 20 mg/kg 6-aminonicotinamide (20). However, before starting our protocol, we performed preliminary experiments in normal dogs to test the tolerability and/or cardiotoxicity of DMSO alone, which is a powerful solvent, and of the dose of 6-aminonicotinamide that we calculated based on the above mentioned assumptions. In four dogs, hemodynamics was measured at baseline and during β-adrenergic stress with 10 μg·kg⁻¹·min⁻¹ of dobutamine, before and after intravenous infusion of 7 ml of DMSO. A similar test was performed in six dogs infused with 80 mg/kg of 6-aminonicotinamide dissolved in 7 ml of DMSO. In this case we also measured cardiac mechanical performance and MVO₂; therefore heart rate was maintained constant by cardiac pacing.

After these preliminary tests, the experimental protocol was started as described in Fig. 1. Experiments were conducted in intact, conscious animals placed on the laboratory table following overnight fasting. HF was induced in 15 dogs by pacing the LV at 210 beats/min for 3 wk and at 240 beats/min for an additional week (27). Dogs were considered in congestive HF when LV end-diastolic pressure was ≥25 mmHg, reflected by clinical signs such as dyspnea and ascites. In 10 HF dogs, a catheter was inserted into the coronary sinus, through a peripheral vein, under X-ray fluoroscopic guidance. Therefore we could withdraw paired blood samples from aorta and coronary sinus. Measurements and blood samples were taken at spontaneous heart rate, with the pacemaker turned off. After baseline hemodynamic measurements, the radioisotopic tracers [9,10-³H]-oleate (0.7 μCi/ml) and [U-¹⁴C]-glucose (20 μCi as a bolus, followed by 0.3 μCi/min) were infused through a peripheral vein to track, respectively, the metabolic fate of FFA and glucose used by cardiac muscle as source of energy (30). Acute hyperglycemia was induced with an infusion of glucose (30 mg·kg⁻¹·min⁻¹ for 5 min, followed by 15 mg·kg⁻¹·min⁻¹ for 20 min) to increase arterial plasma glucose by ~90 mg/dL, thus simulating a marked postprandial peak. Glucose infusion was then stopped to re-establish baseline glycemic levels. At this point, 80 mg/kg of 6-aminonicotinamide dissolved in 7 ml of DMSO was infused intravenously over 15 min. Measurements and blood samples were taken again without and with glucose infusion, as described above.

A group of five chronically instrumented dogs did not undergo cardiac pacing and was used as control to test the effects of glucose infusion on cardiac 8-isoprostane output by normal hearts. MVO₂ and substrate metabolism were not measured in these dogs. They were infused with glucose (30 mg·kg⁻¹·min⁻¹ for 5 min, followed by 15 mg·kg⁻¹·min⁻¹ for 20 min). The infusion was then stopped to re-establish baseline glycemic levels. Dogs belonging to this group were not given 6-aminonicotinamide, so they could later be used in ongoing unrelated studies.

Finally, the remaining five dogs with pacing-induced HF were used to test the effects of adrenergic stimulation, a known cause of cardiac oxidative stress (17), on isoprostane output, and therefore served as positive control: 15 μg·kg⁻¹·min⁻¹ of dobutamine were infused intravenously for 5 min and blood samples collected from aorta and coronary sinus as described above.

At the end of the protocol, the HF dogs were euthanized with 100 mg/kg of pentobarbital sodium. In some of them, the chest was rapidly opened and LV tissue samples immediately harvested and frozen in liquid nitrogen for later biochemical measurements.

Hemodynamic recordings, blood gas analysis, and calculated parameters. The aortic catheter was attached to a strain-gauge transducer to measure aortic pressure. LV pressure was measured using the solid-state pressure gauge. Blood flow in the left circumflex coronary artery was measured with a pulsed Doppler flowmeter (model 100; Triton Technology). LV diameter was measured by connecting the implanted piezoelectric crystals to an ultrasonic dimension gauge. All signals were digitally stored via an analog-digital interface (National Instruments) at a sampling rate of 250 Hz. Digitized data were analyzed offline by custom-made software. The parameters were determined during one respiratory cycle and comprised heart rate, mean aortic pressure, LV end-diastolic, peak systolic and end-systolic pressure, mean blood flow in the left circumflex coronary artery, the maximum and minimum of the first derivative of LV pressure, and end-diastolic and end-systolic LV internal diameters. The area of LV pressure-diameter loops (PDA) was calculated to obtain an index of stroke work (29–31).

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Fig. 1. Experimental protocol.
Blood gases tension was determined using a blood gas analyzer (Instrumentation Laboratory), and oxygen concentration was measured using a hemoglobin analyzer (CO-Oximeter; Instrumentation Laboratory). LV myocardial oxygen consumption (MVO$_2$) per beat was calculated by multiplying the arterial-coronary sinus difference in oxygen content by total mean coronary blood flow, assumed to be twice the mean blood flow in the left circumflex coronary artery, and divided by heart rate (27, 30). Finally, LV external mechanical efficiency was calculated as the ratio PDA/MVO$_2$/beat (29, 31).

**Total and labeled metabolites.** The concentrations of total and labeled FFA, glucose, and lactate, which are three main cardiac energy substrates (34), were determined in arterial and coronary sinus blood samples, as previously described in detail (30). Rapid measurements of total glucose and lactate were obtained from the same blood samples used for blood gas analysis tested with multipurpose cartridges (Instrumentation Laboratory). These measurements were repeated two to three times per sample to verify their consistency. Total FFA concentration in plasma was determined spectrophotometrically.

$^3$H-oleate activity was measured in plasma, whereas $^{14}$C-glucose activity was determined in blood deproteinized with ice-cold 1M perchloric acid (1:2 vol/vol). $^3$H$_2$O and $^{14}$CO$_2$ activities were also measured in plasma and whole blood, respectively.

Mean coronary blood flow and the specific activities of $^3$H-oleate and $^{14}$C-glucose were multiplied, respectively, by the arterial-coronary sinus difference of $^3$H$_2$O and $^{14}$CO$_2$ content and by mean coronary blood flow and then divided by heart rate to calculate the rate of FFA and glucose oxidation per beat. Arterial and coronary sinus concentration of lactate were multiplied by mean coronary blood flow and then divided by heart rate to calculate net chemical lactate uptake per beat.

**Cardiac output of 8-isoprostane.** Isoprostanes are prostaglandin derivatives generated by nonenzymatic, free radical-catalyzed oxidation of arachidonic acid and are considered one of the most sensitive markers of oxidative stress, in vivo (22). Previous experimental and clinical studies have assessed plasma concentrations of 8-isoprostane to monitor cardiac and systemic oxidative stress (4, 5, 14, 15). In the present study, free 8-isoprostane concentrations were measured in plasma samples using the 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s guidelines, as we previously reported (28). To determine the sensitivity of the assay in dog plasma, known amounts of 8-isoprostane (from 10 to 2,000 pg/ml) were mixed with aliquots from the same standard sample. The samples were purified using 8-isoprostane affinity sorbent, dried under nitrogen, and resuspended with EIA buffer before analysis. A standard curve was established by serial dilutions of 8-isoprostane, and the concentration of each sample was calculated from a logistic four-parameter fit of the standard curve. All samples were run in duplicates. The assay was found to be linear in the 10–2,000 pg/ml of 8-isoprostane concentration gradient range tested. The intra-assay coefficient of variation was 14.0%. Cardiac output of 8-isoprostane was calculated as the difference between arterial and coronary sinus concentration multiplied by mean coronary blood flow.

**Biochemical measurements in cardiac tissue.** Frozen LV tissue was pulverized in liquid nitrogen, and homogenates were prepared to measure the activity of G6PD by following the reduction of NADP$^+$ to NADPH. NADPH fluorescence was detected at 340 nm (Ex) and 460 nm (Em) using a Fluoromax microplate fluorescence detector, (BioTek Instruments, Winooski, VT). Moreover, 6P-glucose, a product of the oxPPP, was measured in myocardial tissue by the method of Kaufman et al. We have used these biochemical methods in previous studies (10, 11).

We also tested whether DMSO or 6-aminonicotinamide can directly scavenge superoxide in tissue homogenates. Therefore we tested 1) the effects of DMSO on superoxide production in failing heart tissue and 2) the effects of 6-aminonicotinamide in the presence of xanthine oxidase, a superoxide-generating enzyme that does not need NADPH provided by the oxPPP. LV tissue was prepared and tested with lucigenin for superoxide measurements as previously described by us (10). After pulverization in liquid nitrogen, homogenates were prepared in MOPS (50 mmol/l)-sucrose (250 mmol/l) buffer at pH 7.4. Only freshly prepared homogenates were used for biochemical assays. The homogenates were brought to a final volume of 50 μl. The following five experimental conditions (n = 4 for each condition) were then obtained, with a 30-min incubation time at 37°C for all of them: 1) homogenates of normal heart tissue incubated with no addition of any drug (control); 2) homogenates of failing heart tissue incubated with no addition of any drug (HF); 3) homogenates of failing heart tissue incubated with 1% DMSO (HF + DMSO); 4) homogenates of normal heart tissue incubated with xanthine oxidase (100 IU) + purine (1 mM) (XO); 5) homogenates of normal heart tissue incubated with xanthine oxidase (100 IU) + purine (1 mM) + 6-aminonicotinamide (1 mM) dissolved in DMSO (XO + 6-aminonicotinamide). In experiments 3 and 5, the final concentration of DMSO was 1%, therefore approximately five times higher than the estimated in vivo concentrations in dog tissues.

After incubation, 20 μl of samples were placed in plastic scintillation vials containing 5 μM lucigenin for the detection of superoxide in a final volume of 1 ml of air-equilibrated Krebs solution buffered with 10 mmol/l HEPES-NaOH (pH 7.4). Lucigenin chemiluminescence was expressed in arbitrary units.

**Statistical analysis.** Data are presented as means ± SE. Statistical analysis was performed by employing commercially available software (SigmaStat 3.0). Hemodynamic and metabolic changes at different time points were compared by one-way ANOVA for repeated measures followed by Student-Newman post hoc test. The comparison of cardiac isoprostane output in control versus HF was performed with two-way ANOVA followed by Student-Newman post hoc test. The sample size used for statistical analysis of PDA, coronary flow, cardiac metabolism, and isoprostane output varied due to random technical problems, including distorted pressure-diameter loops, defective coronary flow probes, and difficult blood sampling from the coronary sinus. The comparison of biochemical data from in vitro experiments was performed using a one-way ANOVA followed by Student-Newman post hoc test. For all the statistical analyses, significance was accepted at P < 0.05.

**RESULTS**

In HF, arterial glycemic levels increased significantly by ~90 mg/dl during glucose infusion (Table 1). We were able to re-establish baseline glucose levels before administering 6-aminonicotinamide. After oxPPP inhibition, glucose infusion at the same dose induced glycemic levels not significantly different compared with the first infusion. In control dogs, glucose infusion raised glycemia from a baseline value of 87.6 ± 5.0 to 198.3 ± 1.0 mg/dl (P < 0.05).

**Hemodynamics.** In normal dogs tested with DMSO alone or with 6-aminonicotinamide, we did not observe any significant hemodynamic change, both at baseline and during dobutamine infusion (Table 2). If anything, there was a trend toward the increase in systolic pressure and coronary blood flow. In HF, glucose infusion did not affect hemodynamics under basal conditions, but significantly improved LV systolic pressure, dP/dt$_{max}$, and coronary blood flow after oxPPP inhibition (Fig. 2). LV end-diastolic diameter, an index of preload, was 41.0 ± 1.0 mm at baseline and did not change significantly throughout the experiment (data not shown). In control dogs, glucose infusion did not significantly alter hemodynamics (data not shown). In HF dogs infused with 15 μg·kg$^{-1}$·min$^{-1}$ of dobutamine (n = 5), heart rate increased from 113.0 ± 7.4 to 131.0 ± 9.3 beats/min and dP/dt$_{max}$ from 1,561 ± 120 to 2,343 ± 246 mmHg/s (all P < 0.05).
Cardiac work, MVO₂, and mechanical efficiency. In normal dogs tested with 6-aminonicotinamide, we did not observe any significant change in LV stroke work, as indicated by PDA, and no change in MVO₂ and in mechanical efficiency, both at baseline and during dobutamine infusion (Table 2). In HF, glucose infusion after oxPPP inhibition enhanced LV stroke work, as indicated by PDA (Fig. 3), with a parallel increase in MVO₂. Consistently, the PDA-to-MVO₂ ratio, an index of LV mechanical efficiency, did not display significant changes.

Cardiac energy substrate metabolism. Despite similar glucose availability, the oxidation of this substrate by cardiac muscle was significantly higher after oxPPP inhibition (Fig. 4). As expected, arterial FFA concentration fell markedly and significantly during glucose infusion, and we could not re-obtain the baseline values before administering 6-aminonicotinamide (Table 1); however, their oxidation by the heart did not change significantly throughout the experiment (Fig. 4). Arterial lactate concentration (Table 1) and cardiac uptake (Fig. 4) did not change significantly at any time point.

Cardiac output of 8-isoprostane. 8-Isoprostane significantly increased by more than twofold in response to glucose infusion in HF, but not in control dogs (Fig. 5), despite similarly high glycemic levels. Such change was rapidly reversed in HF when the baseline glycemic levels were re-established. oxPPP inhibition completely prevented the increase in 8-isoprostane output in response to glucose infusion. Despite a clear trend, no significant difference was found at baseline between control and HF, likely due to the relatively small sample size of the two groups. However, when we pooled together samples taken at baseline from the two HF groups (glucose infusion and dobutamine infusion to reach n = 10) and we increased the sample size of control dogs (n = 9), by adding plasma samples from 4 other normal dogs enrolled in different protocols, we found that basal isoprostane output was significantly higher in HF (1022.0 ± 497.60 pmol/min) than in control dogs (333.64 ± 60.31 pmol/min). Finally, in HF dogs infused with dobutamine (n = 5), cardiac isoprostane output increased from a baseline value of 1054.5 ± 235.47 pmol/min to 3531.6 ± 796.0 pmol/min (P < 0.05).

G6PD activity and 6P-glucuronate concentration. Measurements were performed in four LV tissue samples harvested from HF hearts treated with 6-aminonicotinamide and in four samples harvested, using the same post euthanasia procedure, from nontreated HF. G6PD activity was 1.60 ± 0.41 mmol·min⁻¹·mg⁻¹ of protein in treated versus nontreated failing hearts (N.S.). However, 6P-glucuronate concentration was 39.70 ± 8.84 vs. 76.20 ± 11.91 pmol/mg of protein in treated versus nontreated failing hearts (P < 0.05), indicating a significant reduction of this intermediate product after inhibition of the oxPPP by 6-aminonicotinamide.

Table 1. Arterial concentrations of glucose, free fatty acids, and lactate during glucose infusion in dogs with heart failure

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Glucose</th>
<th>Re-Baseline</th>
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<tr>
<td>Glucose arterial concentration, mg/dL</td>
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<tr>
<td>Pre 6-AN</td>
<td>84.66 ± 4.81</td>
<td>173.58 ± 15.15*</td>
<td>104.16 ± 16.80</td>
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<tr>
<td>Post 6-AN</td>
<td>77.50 ± 5.54</td>
<td>178.17 ± 6.79**</td>
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<td>Free fatty acid arterial concentration, mM</td>
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<tr>
<td>Pre 6-AN</td>
<td>0.42 ± 0.08</td>
<td>0.17 ± 0.04*</td>
<td>0.10 ± 0.04</td>
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<tr>
<td>Post 6-AN</td>
<td>0.21 ± 0.05*</td>
<td>0.13 ± 0.03*</td>
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<tr>
<td>Lactate arterial concentration, mM</td>
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<tr>
<td>Pre 6-AN</td>
<td>0.72 ± 0.16</td>
<td>0.97 ± 0.18</td>
<td>1.10 ± 0.97</td>
</tr>
<tr>
<td>Post 6-AN</td>
<td>0.90 ± 0.17</td>
<td>0.78 ± 0.14</td>
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</table>

Values are means ± SE; n = 6. 6-AN, 6-aminonicotinamide. *P < 0.05 vs. baseline 6-AN; **P < 0.05 vs. baseline after 6-AN.

Table 2. Cardiac functional changes during mild β-adrenergic stress before and after administration of DMSO alone (n = 4) or DMSO + 6-AN (n = 5)

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate</th>
<th>Mean Coronary Flow</th>
<th>Systolic Blood Pressure</th>
<th>Maximum First Derivative of Left Ventricular Pressure</th>
<th>Pressure Diameter Area</th>
<th>MVO₂/Beat</th>
<th>Pressure Diameter Area/MVO₂/Beat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>86.5 ± 2.4</td>
<td>33.8 ± 3.3</td>
<td>136.1 ± 13.9</td>
<td>2961.0 ± 423.5</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Dobutamine 10</td>
<td>86.8 ± 5.1</td>
<td>37.5 ± 4.5</td>
<td>147.0 ± 13.7</td>
<td>3787.7 ± 482.5*</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>µg·kg⁻¹·min⁻¹</td>
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<tr>
<td>Re-Baseline</td>
<td>84.0 ± 4.1</td>
<td>33.1 ± 2.8</td>
<td>130.8 ± 6.3</td>
<td>2828.9 ± 256.2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Baseline DMSO</td>
<td>85.7 ± 5.0</td>
<td>38.1 ± 4.1</td>
<td>146.3 ± 10.5</td>
<td>2921.5 ± 253.6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>Dobutamine 10</td>
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<tr>
<td>µg·kg⁻¹·min⁻¹ DMSO</td>
<td>90.0 ± 5.1</td>
<td>50.6 ± 7.9</td>
<td>159.0 ± 15.3</td>
<td>3856.6 ± 354.8*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Baseline</td>
<td>128.3 ± 10.7</td>
<td>35.4 ± 2.3</td>
<td>133.3 ± 9.4</td>
<td>2827.8 ± 314.1</td>
<td>693.4 ± 61.0</td>
<td>0.024 ± 0.000</td>
<td>232.2 ± 19.9</td>
</tr>
<tr>
<td>Dobutamine 10</td>
<td>126.8 ± 5.7</td>
<td>45.9 ± 3.2</td>
<td>130.0 ± 5.9</td>
<td>3845.9 ± 228.3*</td>
<td>853.5 ± 56.7*</td>
<td>0.03 ± 0.003</td>
<td>244.8 ± 42.9</td>
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<td>µg·kg⁻¹·min⁻¹</td>
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<tr>
<td>Re-Baseline</td>
<td>132.5 ± 7.4</td>
<td>34.0 ± 3.2</td>
<td>122.9 ± 3.4</td>
<td>2555.0 ± 190.0</td>
<td>577.8 ± 36.3</td>
<td>0.022 ± 0.003</td>
<td>231.9 ± 28.8</td>
</tr>
<tr>
<td>Baseline DMSO</td>
<td>130.8 ± 9.3</td>
<td>33.2 ± 2.5</td>
<td>124.7 ± 6.9</td>
<td>2435.3 ± 226.1</td>
<td>535.6 ± 61.0</td>
<td>0.020 ± 0.002</td>
<td>240.6 ± 52.5</td>
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<tr>
<td>Dobutamine 10</td>
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</tr>
<tr>
<td>µg·kg⁻¹·min⁻¹ 6-AN</td>
<td>124.6 ± 7.2</td>
<td>44.2 ± 5.0</td>
<td>134.7 ± 8.0</td>
<td>3875.3 ± 405.9*</td>
<td>816.8 ± 59.5*</td>
<td>0.027 ± 0.003</td>
<td>253.9 ± 40.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. In the group tested 6-AN, heart rate was maintained constant throughout the experiment by cardiac pacing. *P < 0.05 vs. baseline; **P < 0.05 vs. Re-Baseline. N.D., not determined.
**PENTOSE PHOSPHATE PATHWAY IN THE FAILING HEART**

**Fig. 2.** Changes in heart rate (HR), maximum first derivative of left ventricular pressure (dP/dt<sub>max</sub>), left ventricular systolic pressure (LVSP), and mean coronary blood flow (MCBF) in the left circumflex coronary artery during glucose infusion, before (●) and after (○) inhibition of the oxidative pentose phosphate pathway (oxPPP). Data are presented as means ± SE; n = 10. *P < 0.05 vs. baseline before 6-aminonicotinamide (6-AN); **P < 0.05 vs. baseline after 6-AN; #P < 0.05 vs. corresponding time point before 6-AN.

**DISCUSSION**

G6PD is markedly upregulated in the failing heart (10, 11, 18), and our previous studies in crude myocardial homogenates from dogs or humans with HF suggest that oxPPP-derived NADPH fuels pro-oxidant systems (10, 11). Very recently, other authors have proposed a similar process to explain, at least in part, the cardiac oxidative stress in mice with defective glycolytic pathway and aortic constriction (35). The present study provides the first in vivo assessment of the pathophysiological relevance of this biochemical mechanism. Our results indicate that oxPPP hyperactivity is causally linked to oxidative stress in the failing heart during physiological changes such as increases in arterial glucose concentration. The two main novel findings were that 1) in HF blood glucose concentrations mimicking postprandial peaks caused approximately twofold increase in cardiac 8-isoprostane output, a marker of oxidative stress, which was completely prevented by oxPPP inhibition and 2) during glucose infusion, the normalization of 8-isoprostane output was associated to an improvement in cardiac work, oxygen consumption, and carbohydrate oxidation, suggesting that the excess generation of reactive oxygen species due to oxPPP hyperactivity limits energy production and conversion into mechanical performance.

The effectiveness of oxPPP inhibition by 6-aminonicotinamide was confirmed by the reduced concentration of 6P-gluconate in LV tissue samples, whereas G6PD activity was not significantly changed. The latter result can be explained considering that 6-aminonicotinamide is a competitive inhibitor; therefore, if given in vivo, its effect tends to disappear after ex vivo tissue processing. Of note, a previous NMR study in mice (20) documented, in vivo, a marked inhibition of the oxPPP by 6-aminonicotinamide given at a dose four times lower than the one used by us. It is also important to emphasize that we likely obtained only a partial inhibition of the oxPPP, sufficient to prevent the detrimental excess NADPH, while maintaining enough reducing equivalents to regenerate the cytosolic anti-oxidant systems. In fact, the dose of 6-aminonicotinamide we used in dogs did not cause harmful effects in normal hearts, even during β-adrenergic stress, whereas a complete oxPPP inhibition could have proven markedly cardiodepressant, as suggested by previous studies in isolated cardiomyocytes (16).

Experimental and clinical studies have shown that cardiac FFA oxidation, the main source of energy in the normal heart during fasting (34), is downregulated in HF, with consequent reliance on higher glucose consumption (3, 23, 26, 27). The debated pathophysiological implications of this metabolic alteration can be considered under a new perspective in the light of the present findings. There are no direct methods available to measure separately, in vivo, the rate of glucose flux through the oxPPP versus the glycolytic-oxidative pathway. The most reliable assessment of oxPPP activity under the various experimental conditions would have required myocardial tissue sampling after euthanasia to quantitate intermediate glucose metabolites (38) at each time point, an approach necessitating the use of an unrealistic number of dogs. However, based on the known metabolic alterations of the failing heart, which encompass elevated rates of glucose consumption and more than doubled tissue concentrations of 6P-gluconate (10, 26, 27), it is conceivable that an abnormally high fraction of glucose is directed into the upregulated oxPPP. We enhanced glucose delivery to the heart to further stimulate myocardial carbohydrate utilization. Glycemic levels were purposely raised up to values corresponding to pronounced physiological postprandial peaks to render the functional and biochemical
changes more clearly detectable. The marked elevation of cardiac 8-isoprostane output, which was prevented by 6-aminonicotinamide, strongly suggests that glucose infusion worsened cardiac oxidative stress, likely due to excess NADPH generated by G6PD and 6-phosphogluconate dehydrogenase. We have previously provided direct evidence of this mechanism, ex vivo: the addition of NADPH, but not NADH, to canine or human failing heart tissue homogenates caused a marked increase in superoxide production (10, 11). A similar mechanism has been proposed by other authors to explain the detrimental role of oxPPP in cardiac ischemia/reperfusion (38). Moreover, it has been shown in cultured cardiomyocytes that NADPH oxidase is activated by prolonged exposure to 21 mM glucose, but cannot generate superoxide without oxPPP-derived NADPH (1). Other authors have used plasma 8-isoprostane as a sensitive in vivo marker of cardiovascular oxidative stress (4, 5, 14, 15), and we confirm its high reliability in vivo, with clearly detectable differences between HF and control at the steady state and marked changes in response to acute events. In fact, notable increases in 8-isoprostane output were found also in the HF group infused with dobutamine infusion and that served as positive control. It has been well documented by other authors that catecholamine catabolism by monoamine oxidases produces hydrogen peroxide (17). To our knowledge, the present results provide the first direct evidence,
in vivo, that the inotropic effects of catecholamines are associated with cardiac generation of reactive oxygen species. They also indicate that, in HF, cardiac isoprostane output increases in response to glycemic peaks or to moderate adrenergic stimulation (~50% increase in dP/dt\textsubscript{max}) by a similar order of magnitude (2- to 3-fold).

In our opinion, our findings have high clinical relevance, since they indicate that a physiological, cyclic event such as the postprandial raise in plasma glucose is sufficient, per se, to rapidly accelerate the production of reactive oxygen species in the failing, but not in the normal, heart. Such recurrent surges add to the basal cardiac oxidative stress that is notoriously high in HF (6, 8, 13, 24, 31) as confirmed by the present data of baseline 8-isoprostane output. Interestingly, oxPPP inhibition enhanced cardiac glucose oxidation and, consistently, oxygen consumption and stroke work, but only during acute hyperglycemia, i.e., only when more energy substrate was supplied to the heart. This improvement could not be explained by significant differences in arterial concentrations of glucose and FFA before and after 6-aminonicotinamide and was likely due to the normalization of oxidative stress and attenuation of the characteristic metabolic rigidity of the failing heart (26), which limits substrate oxidation and, in turn, conversion of chemical energy into contractile work. However, with the technology employed in the present study we could not determine whether this increase in MVO\textsubscript{2} ultimately re-established a more physiological phosphocreatine/ATP ratio, known to be reduced in the failing heart (9). Because the attenuation of oxidative stress enhanced also coronary blood flow during glucose infusion, we cannot exclude that part of the beneficial effects were due to the improved myocardial perfusion. In fact, under pathological conditions isoprostanes can cause coronary constriction (37). A previous study in tachycardia-induced HF has shown the profound negative impact of oxidative stress on the mechanoen-ergetic coupling expressed by the PDA-to-MVO\textsubscript{2} ratio (31). In the present study, we found that oxPPP inhibition enhances both stroke work and oxygen consumption.

The ensuing question is whether a sustained inhibition of the oxPPP, possibly started at early stages of the disease, would keep oxidative stress low and therefore delay the progression of HF. Ideally, G6PD activity should be maintained within the physiological range, sufficient to feed the anti-oxidant system, but not so much as to generate an excess of NADPH that could be used by pro-oxidant enzymes. Unfortunately, the currently available pharmacological agents and animal models are not adequate to test this hypothesis. Chronic administration of 6-aminonicotinamide is toxic in dogs (21), likely due to the systemic inhibition of the vital enzymes of the oxPPP. To circumvent this problem, in a recent study we induced HF by coronary ligation or transverse aortic constriction in mice harboring a genetic downregulation of G6PD (40% of normal activity) compatible with normal life (12). The results were surprising: mice with G6PD mutation displayed a modest worsening of the early stages of HF progression, hence no major negative or positive differences compared with wild-type mice. Also in this case, however, the model was not ideal, since mutant mice have an inborn defect, which might activate numerous compensatory mechanisms.

An important limitation we should acknowledge is the absence of a group of HF dogs tested with the infusion of DMSO to exclude confounding effects of the vehicle alone. However, because in our preliminary tests we did not observe major effects of a similar volume of DMSO in normal dogs, even during β-adrenergic stress, and considering also our necessity to use the minimum possible number of chronically instrumented dogs, we deemed reasonable not to perform additional experiments with the vehicle alone. Moreover, our findings in tissue samples seem to rule out potential, direct anti-oxidant effects of DMSO at concentrations calculated, very conserva-
vatively, based on the total amount of vehicle infused in vivo. We also acknowledge that, although the main focus of our study was on heart failure, a thorough investigation of the metabolic effects of oxPPP inhibition during hyperglycemia in normal dogs could have been very interesting. Also in this case, we based our decision in part on negative results (no significant changes in cardiac isoprostane production during glucose infusion in normal dogs) and in part on the necessity to use the minimum possible number of chronically instrumented dogs.

Conclusions

In conclusion, our study has identified, in vivo, a novel link between oxPPP activity and oxidative stress in normal and failing hearts. The same metabolic pathway that is otherwise essential for cell survival might contribute to organ damage in HF, suggesting that interventions aimed at modulating cardiac glucose metabolism could be used to indirectly mitigate oxidative stress in the failing heart.

![Cardiac Isoprostane Output](fig5.png)

**Fig. 5.** Changes in cardiac 8-isoprostane output during glucose infusion before and after inhibition of the oxPPP. Data are presented as means ± SE. *P < 0.05 vs. baseline before 6-AN; †P < 0.05 vs. corresponding time point before 6-AN; ‡P < 0.05 vs. control. HF, heart failure. Re-Base, re-baseline.

<table>
<thead>
<tr>
<th></th>
<th>Lucigenin chemiluminescence (arbitrary units)</th>
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<tbody>
<tr>
<td>Control</td>
<td>482 ± 25</td>
</tr>
<tr>
<td>Heart failure</td>
<td>1,080 ± 300*</td>
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<tr>
<td>Heart failure + DMSO</td>
<td>1,110 ± 285*</td>
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<tr>
<td>Xanthine oxidase</td>
<td>5,679 ± 1,509*</td>
</tr>
<tr>
<td>Xanthine oxidase + 6-AN</td>
<td>5,376 ± 1,792*</td>
</tr>
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Values are means ± SE; n = 4/group. *P < 0.05 vs. control.

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**Table 3. Direct effects of DMSO or 6-AN on superoxide production in tissue homogenates**

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


