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Title Page

Chronic Activation of PPARα with Fenofibrate Prevents Alterations in Cardiac Metabolic Phenotype without Changing the Onset of Decompensation in Pacing-Induced Heart Failure

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Running Title Page

Running title: Fenofibrate alters metabolism in the failing heart

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Abbreviations:

HF, heart failure; Feno, fenofibrate; FFA, free fatty acid; PPARα, peroxisome proliferators-activated receptor-α; LV, left ventricle; MCAD, medium chain acyl-CoA dehydrogenase.

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ABSTRACT

Severe heart failure (HF) is characterized by profound alterations in cardiac metabolic phenotype, with downregulation of the free fatty acid (FFA) oxidative pathway and marked increase in glucose oxidation. We tested whether fenofibrate, a pharmacological agonist of PPARα, the nuclear receptor that activates the expression of enzymes involved in FFA oxidation, can prevent metabolic alterations and modify the progression of HF. We administered 160 mg/day p.o. of fenofibrate to eight chronically instrumented dogs over the entire period of high frequency left ventricular pacing (HF+Feno). Eight additional HF dogs were not treated, and eight normal dogs were used as a control. ^{3}H-oleate and ^{14}C-glucose were infused intravenously to measure the rate of substrate oxidation. At 21 days of pacing, left ventricular end-diastolic pressure was significantly lower in HF+Feno (14.1±1.6 mmHg) compared to HF (18.7±1.3 mmHg), but increased up to 25±2 mmHg, indicating end-stage failure, in both groups after 29±2 days of pacing. FFA oxidation was reduced by 40% and glucose oxidation was increased by 150% in HF compared to control, changes that were prevented by fenofibrate. Consistently, the activity of myocardial medium chain acyl-CoA dehydrogenase, a marker enzyme of the FFA β-oxidation pathway, was reduced in HF vs control (1.46±0.25 vs 2.42±0.24 μmol/min/gww, $p<0.05$), but not in HF+Fenof (1.85±0.18 μmol/min/gww, NS vs control). Thus preventing changes in myocardial substrate metabolism in the failing heart causes a modest improvement of cardiac function during the progression of the disease, with no effects on the onset of decompensation.

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Introduction

The cardiac metabolic phenotype undergoes profound alterations during heart failure, including defective energy production, lower mechanical efficiency and a partial shift in energy substrate utilization (Stanley et al., 2005). Oxidation of free fatty acids (FFA), which constitutes the preferential energy source for the normal heart, decreases in overt heart failure, whereas glucose oxidation markedly increases. The mechanisms underlying this phenomenon are numerous and complex. There is reduced myocardial expression and activity of key enzymes of the FFA oxidative pathway in different models of human as well as experimental heart failure (Sack et al., 1996; Osorio et al., 2002). The expression of these enzymes is under the control of the peroxisome proliferators-activated receptor-α (PPARα) and retinoid X receptor-α (RXRα), nuclear receptors that were also found downregulated in the failing heart (Osorio et al., 2002; Karbowska et al., 2003). Whether such alterations in substrate metabolism play a role in the pathophysiological progression of heart failure remains an open question, with obvious implications for new therapeutic strategies based on metabolic modulators (Stanley et al., 2005). It has been proposed that a reduced FFA oxidative capacity is a detrimental event in the progression of cardiac pathologies towards failure (Barger et al., 2000), whereas others have found beneficial effects of pharmacological inhibitors of FFA oxidation (Rupp and Vetter, 2000; Lionetti et al., 2005; Lee et al., 2005; Fragasso et al., 2006) or stimulators of glucose uptake (Bersin et al.,1994; Nikolaidis et al, 2004). Moreover, it has been shown that the reactivation of PPARα by a selective agonist is associated with contractile dysfunction in pressure overload-induced hypertrophy, suggesting that PPARα down-regulation is essential for the maintenance of contractile function of the

hypertrophied heart (Young et al, 2001). The negative effects of PPARα stimulation were not confirmed in a rat model of post-infarction heart failure, in which chronic administration of the PPARα agonist fenofibrate enhanced left ventricular hypertrophy, but did not cause greater deterioration of mechanical and hemodynamic parameters (Morgan et al., 2006). It is possible that different etiologies of HF, such as ischemia, pathological hypertrophy or idiopathic cardiomyopathy, differentially effect cardiac substrate metabolism and respond differently to treatment with a PPARα agonist.

Pacing-induced heart failure has been used for many years as an established model of dilated cardiomyopathy and is characterized by a very predictable time course. We previously found in this model that the inhibition of FFA oxidation causes a significant delay of decompensation and prevents other important molecular alterations (Lionetti et al., 2005), therefore in the present study we tested the hypothesis that a sustained pharmacological activation of PPARα with the specific agonist fenofibrate (Forman et al., 1997) can accelerate the development and progression of HF. Large animal models are particularly advantageous for this type of investigation, in that they allow withdrawal of blood samples from coronary sinus in conscious animals to measure cardiac substrate metabolism *in vivo*. At the end of the experiments, myocardial biopsies from the beating heart were freeze-clamped to quantify the activity and gene expression of key metabolic enzymes.

Methods

Surgical instrumentation and hemodynamic measurements

Twenty-four adult, male, mongrel dogs (25-27 kg) were sedated with acepromazine maleate (1 mg/kg im), anesthetized with pentobarbital sodium (25 mg/kg iv), ventilated with room air and chronically instrumented as previously described (Recchia et al., 1998; Lionetti et al., 2005). A thoracotomy was performed in the left fifth intercostal space. One catheter was placed in the descending thoracic aorta, and a second catheter was placed in the coronary sinus with the tip leading away from the right atrium. A solid-state pressure gauge (P6.5; Konigsberg 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 human, screw-type, unipolar myocardial pacing lead was fixed in the left ventricular (LV) 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 recover fully. 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 (IACUC) of the New York Medical College and conform to the guiding principles for the care and use of laboratory animals published by the National Institutes of Health.

Experimental protocol

Heart failure (HF) was induced in 16 dogs by pacing the left ventricle at 210 bpm for 3 weeks, and then the pacing rate was increased to 240 bpm. This model has a very predictable time course characterized by a phase of compensated cardiac dysfunction,

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with no major alterations in pulmonary gas exchange and no clinical evidence of failure and a final phase of decompensation, whose onset is indicated by a LV end-diastolic pressure of 25 mmHg (Recchia et al., 1998). Eight of the paced dogs received 6.5 mg/kg/day per os of fenofibrate (HF+Feno), a hypolipidemic agent (Adkins et al., 1997) and direct activator of PPARα (Forman et al., 1997; Staels and Fruchart, 2005). Fenofibrate administration was started from the first day of pacing and continued until end-stage failure. Since it was necessary to harvest large cardiac biopsies at the end of the experiment, we used a separate group of 8 normal dogs for control samples of myocardium. Experiments were conducted in the morning in conscious dogs placed on the laboratory table after overnight fasting. Hemodynamic variables were recorded and echocardiographic measurements were performed at baseline, at 3 weeks, which corresponds to compensated failure, and in end-stage HF. The experiments were performed at spontaneous heart rate, with the pacemaker turned off. Dogs were considered in end-stage HF when left ventricular end-diastolic pressure reached 25 mmHg and clinical signs of severe decompensation were observed (Recchia et al., 1998; Osorio et al., 2002). Hemodynamics were recorded and the isotopic tracers [9,10-^{3}H]-oleate (0.7 μCi/min) and [U-^{14}C]-glucose (20 μCi + 0.3 μCi/min) were continuously infused for the duration of the experiment through a peripheral vein to track the metabolic fate of FFA and carbohydrates utilized by cardiac muscle as source of energy (Osorio et al., 2002; Lei et al., 2004). After 40 min of tracer infusion, paired blood samples were withdrawn from the aorta and coronary sinus. In one HF+Feno dog, isotopes were not infused, due to the occlusion of the coronary sinus catheter and the consequent inability to withdraw paired blood samples. At the end of this procedure, the dogs were

anesthetized with 30 mg/kg of sodium pentobarbital i.v., intubated and ventilated. The fifth intercostal space was rapidly opened to harvest a large transmural biopsy (~10 g) from the left ventricular anterior free wall while the heart was still beating. The harvested tissue was immediately freeze-clamped with tongs pre-cooled in liquid nitrogen as previously described (Osorio et al., 2002; Lei et al., 2004; Lionetti et al., 2005). The heart was then removed and weighed to determine the heart mass to body mass ratio.

Hemodynamics, echocardiographic recordings and calculated parameters

The aortic catheter was attached to a P23ID strain-gauge transducer for measurement of aortic pressure. LV pressure was measured using the solid-state pressure gauge. The first derivative of LV pressure, LV dP/dt, was obtained using an operational amplifier (National Semiconductor LM 324). Coronary blood flow was measured with a pulsed Doppler flowmeter (Model 100, Triton Technology). All signals were recorded on an eight-channel direct-writing oscillograph (Gould RS 3800). The analog signals were also stored in computer memory through an analog-digital interface (National Instruments), at a sampling rate of 250 Hz. (Recchia et al., 1998; Osorio et al., 2002). Two-dimensional and M-mode echocardiography was also performed (Sequoia C256). Images were obtained from a right parasternal approach at the mid–papillary muscle level, according to the criteria of the American Society of Echocardiography, as previously described (Lionetti et al., 2005).

Total and labeled metabolites

Oxygen content and total cardiac substrate concentrations were measured in arterial and coronary sinus blood samples. Analysis and calculations were performed as previously described (Osorio et al., 2002; Lei et al., 2004). In brief, blood gases and oxygen content were measured by using a blood gas analyzer and a hemoglobin analyzer, respectively. FFA concentration was determined spectrophotometrically in plasma. Glucose and lactate concentrations were measured in blood deproteinized with ice-cold 1M perchloric acid (1:2 vol/vol) using spectrophotometric enzymatic assays. The concentrations of labeled metabolites were determined in arterial and coronary sinus blood samples. In particular, ^{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). $^{3}H_2O$ and $^{14}CO_2$ activities were also measured in plasma and whole blood, respectively. Myocardial oxygen consumption (MVO_2) was calculated by multiplying the arterial-coronary sinus difference in oxygen content by mean coronary blood flow. The concentration of total and labeled substrate in arterial and coronary blood samples and mean coronary blood flow were used to calculate the rates of FFA, lactate and glucose uptake (μmol/min). 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 in $^{3}H_2O$ and in $^{14}CO_2$ and by mean coronary blood flow to calculate the rates of FFA and glucose oxidation (μmol/min). MVO_2 and rates of substrate consumption were normalized by cardiac weight.

Triglycerides and total cholesterol were measured in duplicate in serum by standard enzymatic techniques (Synchron CX9 Pro, Beckman Coulter, Inc., Fullerton USA).

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Enzyme activities and metabolic products in cardiac tissue

We measured the activity of the citric acid cycle enzyme citrate synthase, a marker of mitochondrial Krebs cycle function, and of medium chain acyl-CoA dehydrogenase (MCAD), a marker enzyme of the fatty acid β-oxidation pathway whose expression is under the control of the nuclear receptor PPARα. These activities were measured in powdered left ventricular tissue as previously described by us (Osorio et al., 2002; Lionetti et al., 2005).

Gene Expression Analysis

RNA was extracted using standard methods and analyzed using reverse transcription followed by real-time quantitative polymerase chain reaction (PCR) for the transcripts of interest, as described previously (Lionetti et al., 2005). Primers and probes for canine MCAD were: 5'-TGGCACGTTCTGATCCAGAT-3' (forward primer), 5'CGCTGGCCCATGTTTAATT-3' (reverse primer) and 5'-FAM AAAGCCTTTACTGGATTCATTGTGGAAGCA-TAMRA-3' (probe). Standard RNA was made for all assays by the T7 polymerase method (Ambion), using total RNA isolated from the dog heart. Transcript levels are expressed as the number of molecules of mRNA per ng of total RNA (as measured by UV spectrophotometry).

Statistical analysis

Data are presented as mean ±standard error of the mean (SEM). Statistical analysis was performed by employing commercially available software (SigmaStat 3.01).

Hemodynamic and metabolic changes at different time points in the same group and differences between groups were compared by one- and two-way ANOVA followed by the Tukey post-hoc test. For all the statistical analyses, significance was accepted at $p<0.05$.

Results

Hemodynamic Alterations

Figure 1, panel A, shows changes in LV end-diastolic pressure (LVEDP). In this model, we consider a LVEDP of 25 mmHg as a hallmark of end-stage failure (Recchia et al., 1998). Chronic fenofibrate administration significantly delayed the increase of LV end-diastolic pressure at three weeks of pacing, however this beneficial effect was only transient and did not further affect the progression towards end-stage failure that occurred at the same time point in both groups. Alterations of other key hemodynamic parameters (panels B-D) followed the typical pattern previously found in this model of failure (Recchia et al., 1998), with a marked reduction in mean arterial pressure, LV systolic pressure and dP/dt_{max}, an index of systolic function. Fenofibrate treatment did not affect these changes. Left circumflex coronary artery blood flow prior to the initiation of pacing was 41.6±2.0 mL/min in HF and 39.4±3.5 mL/min in HF+Feno (N.S.) and did not change over the progression of failure. Spontaneous heart rate increased significantly within each group from 97.2±4.5 to 133.1±5.8 beats/min in HF and from 94.0±6.2 to 121 ±4.9 in HF+Feno, with no difference between groups.

LV Ejection Fraction and Dimensions

In end stage failure, LV ejection fraction was significantly higher in HF+Feno compared to untreated HF (Figure 2A). LV end-diastolic diameter increased whereas wall thickness decreased significantly during the progression of dilated cardiomyopathy, with no significant differences between the two groups (Figure 2B-D). LV end-systolic diameter was not significantly different between the two groups (data not shown).

Consistent with the changes in ejection fraction, we found that the percent shortening in LV diameter during systole in HF and HF+Feno was, respectively, 0.35±0.02 and 0.36±0.03 at baseline (N.S.), 0.22±0.02 and 0.20±0.01 at 21 days (N.S.) and 0.16±0.01 and 0.23±0.03 ($p<0.05$) in end stage failure. The heart weight/body weight ratio was not different among three groups (8.4±0.2 g/kg in normal dogs, 9.0±0.3 g/kg in HF and 8.5±0.4 g/kg in HF+Feno).

Circulating lipids

Circulating triglyceride levels were not affected by the progression of HF, however treatment with fenofibrate resulted in lower triglyceride concentration at 21 days of pacing in and also in end stage failure. (Figure 3). On the other hand, circulating cholesterol was significantly reduced during pacing in the untreated HF dogs, but this decrease was significantly more pronounced in the fenofibrate treated dogs, with a progressive fall from 21 days to end stage failure.

Cardiac metabolism

There were no significant differences in arterial concentrations of FFA or lactate among control, HF, and HF+Feno (Table 1), however plasma glucose was approximately 20% higher in the untreated HF.

MVO_2 was not significantly different among the three experimental groups (Figure 4A), however FFA oxidation was reduced by approximately 40% in HF compared to control (Figure 4B), while glucose oxidation was increased by approximately 2.5 fold in HF

compared to control (Figure 4C). Fenofibrate treatment prevented these metabolic changes. Net lactate uptake was similar among groups (Figure 4D).

Metabolic Enzymes

The activity of the citric acid cycle enzyme citrate synthase was reduced by 33% in HF compared to control hearts, but there was no significant change versus control in the HF+Feno group (Figure 5). The activity of medium chain acyl-CoA dehydrogenase (MCAD) was also significantly decreased in HF compared to control, which was prevented in the HF+Feno group. Similarly, MCAD gene expression was decreased by 55% in HF compared to normal, but was not significantly reduced in the HF+Feno group (Figure 5).

Discussion

The present study shows that a sustained activation of PPARα during pacing-induced heart failure prevents alterations in cardiac FFA and glucose oxidation and the downregulation of medium chain acyl-CoA dehydrogenase (MCAD), one of the key enzymes involved in mitochondrial FFA beta-oxidation cycle, without accelerating the progression towards decompensated heart failure. These results are surprising, since we have previously found that an opposing intervention, i.e. pharmacological inhibition of mitochondrial fatty acid oxidation, delays the onset of decompensation in pacing-induced heart failure (Lionetti et al., 2005). This is the first evidence to suggest that the downregulation of myocardial fatty acid oxidation and acceleration in glucose oxidation in advanced heart failure in not a necessary compensatory mechanism to optimize cardiac energetics, as previously thought.

Fenofibrate is a member of the fibrate class that displays hypolipidemic actions (Adkins and Faulds, 1997; Staels et al., 1998) and is clinically used in patients with hyper-triglyceridemia. To a lesser extent, it can also lower plasma cholesterol levels. The triglyceride-lowering activity of fibrates is attributed to both inhibition of hepatic fatty acid synthesis and increased catabolism of very low density lipoproteins (Staels et al., 1998), likely mediated through the interaction with nuclear receptor PPARα (Staels et al., 1998; Gervois et al., 2000). Once activated by fenofibrate, PPARα forms a complex with retinoid X receptor-α and with the co-activator PGC-1 and binds sequence-specific target elements of a number of gene promoters (Vega et al., 2000; Berger and Moller, 2002). In the present study, we administered fenofibrate at the daily dose that is normally used in humans, therefore, given the difference in body size, the dogs received approximately

threefold the amount recommended for clinical use. Other studies in animals have employed a much higher dosage of fenofibrate (Morgan et al., 2006), but we chose to maintain a regimen closer to the human therapeutical range. The efficacy of the dose employed in our study is demonstrated by the significant effects on lipid plasma levels and on cardiac energy substrate metabolism in treated, compared to untreated HF dogs. After 21 days of pacing, fenofibrate lowered triglyceride and cholesterol by 40% and 15%, respectively, very close to the effects recently found by other authors who administered 10 mg/kg/day to dogs for 15 days (Serisier et al., 2006). Moreover, in HF+Feno circulating cholesterol was decreased further in end stage failure compared to 21 days. Although our study was not designed to assess the pharmacokinetics of fenofibrate, taken together these data indicate that the dose used in the present study was sufficient to reach pharmacological efficacy over the entire period of pacing.

The main goal of our study was to prevent the downregulation of the FFA oxidative pathway by activating PPARα. Cardiac mRNA level and activity of MCAD, a key enzyme of mitochondrial fatty acid β-oxidation, were decreased by heart failure as previously shown in this model (Osorio et al., 2002; Lionetti et al., 2005) and humans (Sack et al., 1996), but was not significantly different from control dogs in fenofibrate-treated HF animals, consistent with a preserved activation state of PPARα. Since there were modest differences in plasma FFA concentrations among groups, these results strongly suggest that the greater rate of FFA oxidation in the HF+Feno group compared to the untreated HF animals was due to direct myocardial effects of fenofibrate. We cannot exclude, however, other PPARα-independent mechanisms of action accounting for the observed metabolic effects. For instance, it is known that fibrates can cause a

rightward shift of the hemoglobin dissociation curve (Wootton, 1984). This might have enhanced myocardial oxygen supply and thus prevented metabolic remodeling during the pacing protocol, when cardiac muscle was under a constant elevated need for energy.

The pathophysiological significance of the altered cardiac energy substrate utilization, observed in human as well as experimental heart failure, is the object of much debate and investigation (Stanley et al., 2005). It has been hypothesized that there is a link between metabolic pathways and mechanisms of myocyte adaptation to chronic pathological stress. Furthermore, pharmacological modulators have been proposed as a new class of drugs for the treatment of heart failure (Nikolaidis et al., 2004; Lee et al., 2005; Fragasso et al., 2006). Results from previous studies suggest that the shift towards higher myocardial glucose utilization may function as a beneficial adaptation in heart failure and pathological hypertrophy (Stanley et al., 2005; Liao et al., 2002). We recently found that a sustained blockade of CPT-I delayed by one week the progression of pacing-induced heart failure and markedly attenuated left ventricular remodeling (Lionetti et al., 2005). Rupp and colleagues described a beneficial effect of CPT-I blockers in limiting the evolution of pathological hypertrophy towards failure (Rupp and Vetter, 2000). Based on these results, we expected that a pharmacological intervention aimed at preventing the downregulation of FFA oxidation pathway would accelerate the progression of heart failure, yet this was not the case. On the contrary, fenofibrate limited the increase in LV end-diastolic pressure at 21 days of pacing, indicating a slower progression of heart failure at least during the compensated phase. Moreover, in end stage failure, the decrease in ejection fraction and percent shortening of LV diameter without significant differences in end diastolic diameter and mean arterial pressure between the

two groups indicate that fenofibrate partially preserved cardiac contractility. Despite this beneficial effect on systolic function, fenofibrate did not delay the onset of overt congestive failure. One possibility is that PPARα activation does not affect molecular mechanisms responsible for the development of dilated cardiomyopathy. Perhaps the beneficial effects previously found with CPT-1 inhibitors are related to the inhibition of this specific enzyme, rather than to a generic reduction of FFA oxidation. However, our results are consistent with the lack of effects of fenofibrate on cardiac function and left ventricular remodeling described in a rat model of post-infarct heart failure (Morgan et al., 2006). In that study, fenofibrate was administered at the dose of 150 mg/kg/day and, although the rate of cardiac FFA and carbohydrate oxidation was not determined, the authors found a more dramatic upregulation of MCAD mRNA and activity in fenofibrate-treated infarcted hearts, indicative of a potentiation of the fatty acid oxidation pathway.

In conclusion, the PPARα agonist fenofibrate can effectively prevent changes in myocardial substrate metabolism that occur in pacing-induced heart failure. Such a remarkable effect on energy substrate selection is accompanied by a modest improvement of cardiac function during the progression of the disease, whereas PPARα activation does not affect the time to terminal decompensation. These findings prompt new questions about the implications of cardiac metabolic alterations in the pathophysiology of dilated cardiomyopathy.

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Footnotes

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Legends For Figures

Figure 1. Changes in LV end-diastolic pressure (LVEDP, panel A), mean aortic pressure (MAP, panel B), LV systolic pressure (LVSP, panel C) and dP/dt_{max} (panel D) during the progression of pacing induced HF. n= 8 for both groups. Data are mean ±SEM. * $p<0.05$ vs day 0 (baseline), # $p<0.05$ between untreated HF and HF+Feno.

Figure 2. Changes in LV ejection fraction (LVEF, panel A), LV end-diastolic diameter (LVEDD, panel B), end-diastolic thickness of the LV free wall (LVFWT, panel C) and end-diastolic thickness of the interventricular septum (IVST, panel D) during the progression of pacing induced HF. n=8 for both groups. Data are mean ±SEM. *$P<0.05$ vs day 0 (baseline). # $p<0.05$ between untreated HF and HF+Feno.

Figure 3. Changes in arterial concentration of triglyceride (panel A) and cholesterol (panel B) during the progression of pacing induced HF. n= 6 for both groups. Data are mean ±SEM. * $p<0.05$ vs day 0 (baseline), † $p<0.05$ vs 21 days of pacing, # $p<0.05$ between untreated HF and HF+Feno

Figure 4. Changes in MVO_2 and cardiac FFA oxidation, glucose oxidation and net lactate uptake in HF and HF+Feno compared to normal dogs (control). N=8 for control and HF and n=7 for HF+Feno. Data are mean ±SEM. * $p<0.05$ vs control; # $p<0.05$ between untreated HF and HF+Feno.

Figure 5. Activity of citrate synthase (CS) and mRNA expression and activity for medium chain acyl-CoA dehydrogenase (MCAD). N=8 for control and HF and n=7 for HF+Feno. Data are mean ±SEM. * $p<0.05$ vs control.

Table 1. Arterial concentrations of cardiac metabolic substrates and lipids

	Control	Heart Failure	Heart Failure + Fenofibrate
FFA (mM)	0.69 ± 0.05	0.45±0.04	0.69±0.15
Glucose (mM)	4.42 ± 0.20	5.32 ± 0.26*	4.60±0.27
Lactate (mM)	0.83 ± 0.08	0.88 ± 0.11	0.63±0.08

*p<0.05 vs control.

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Figure 1

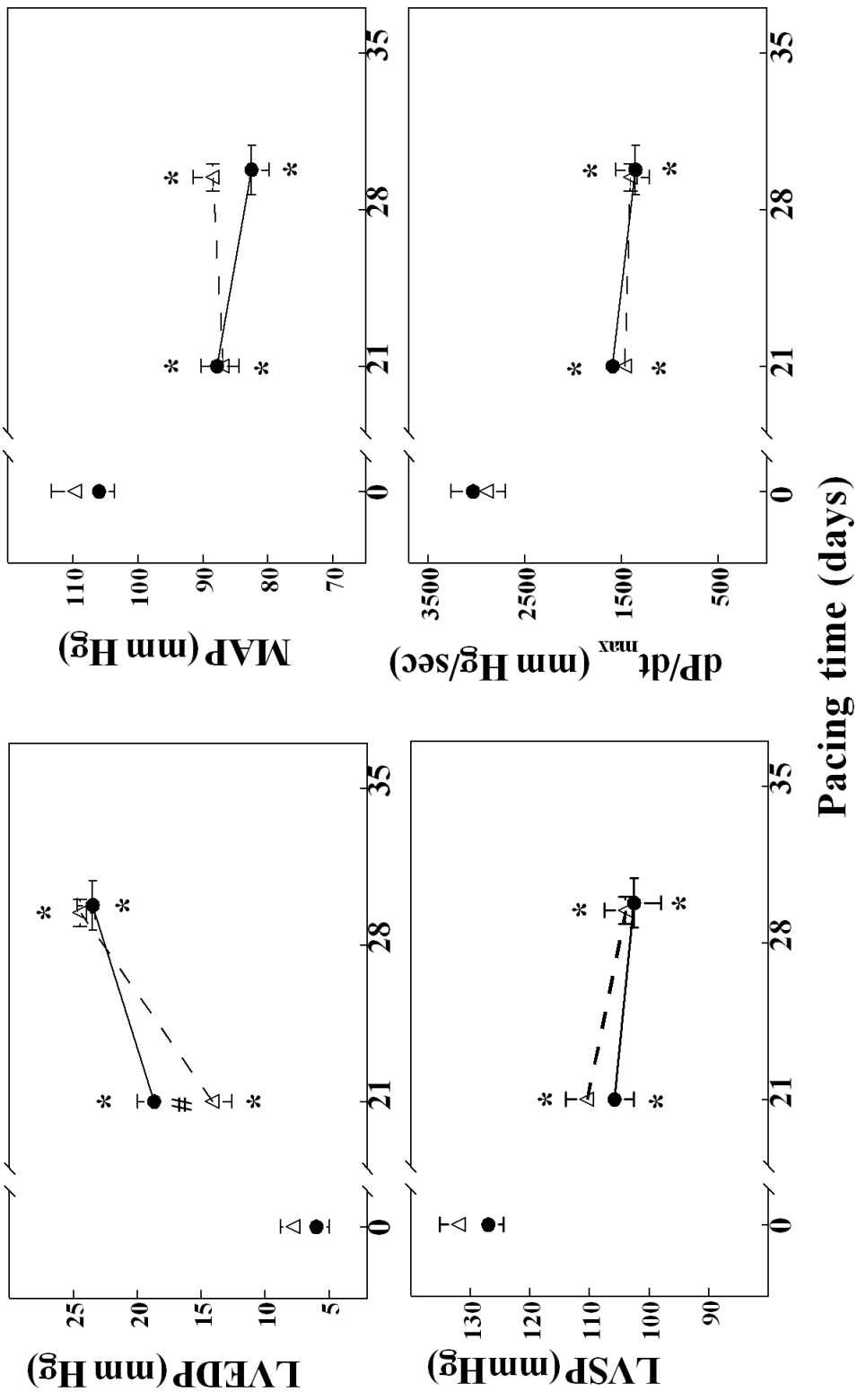

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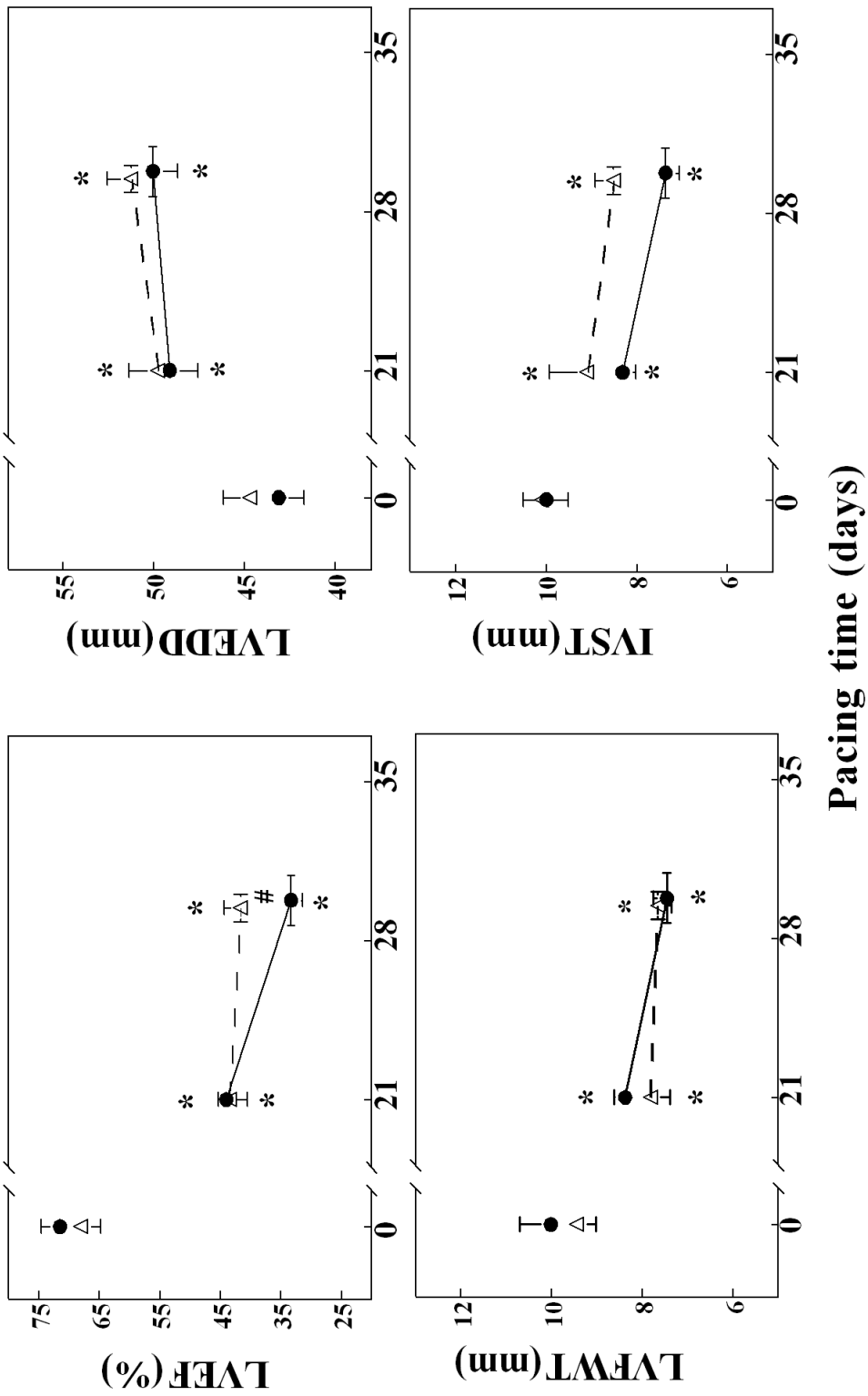
Figure 2



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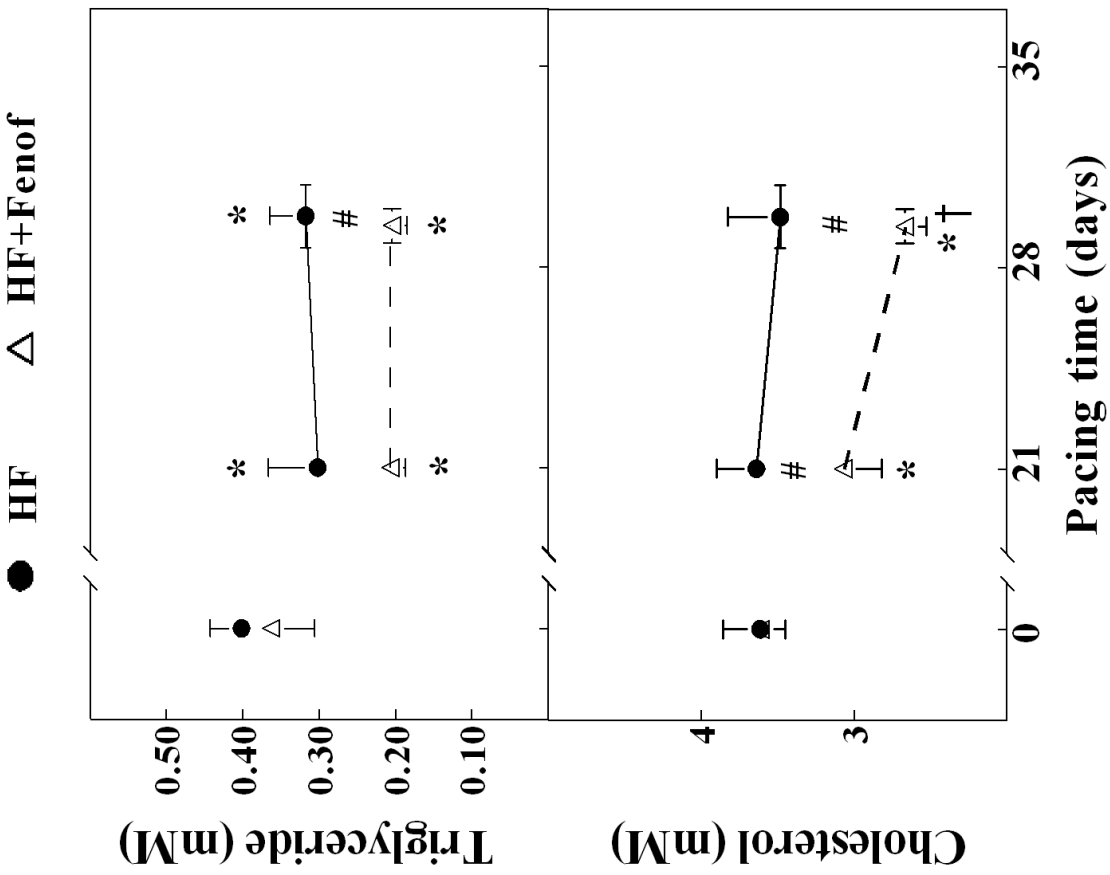

Figure 3

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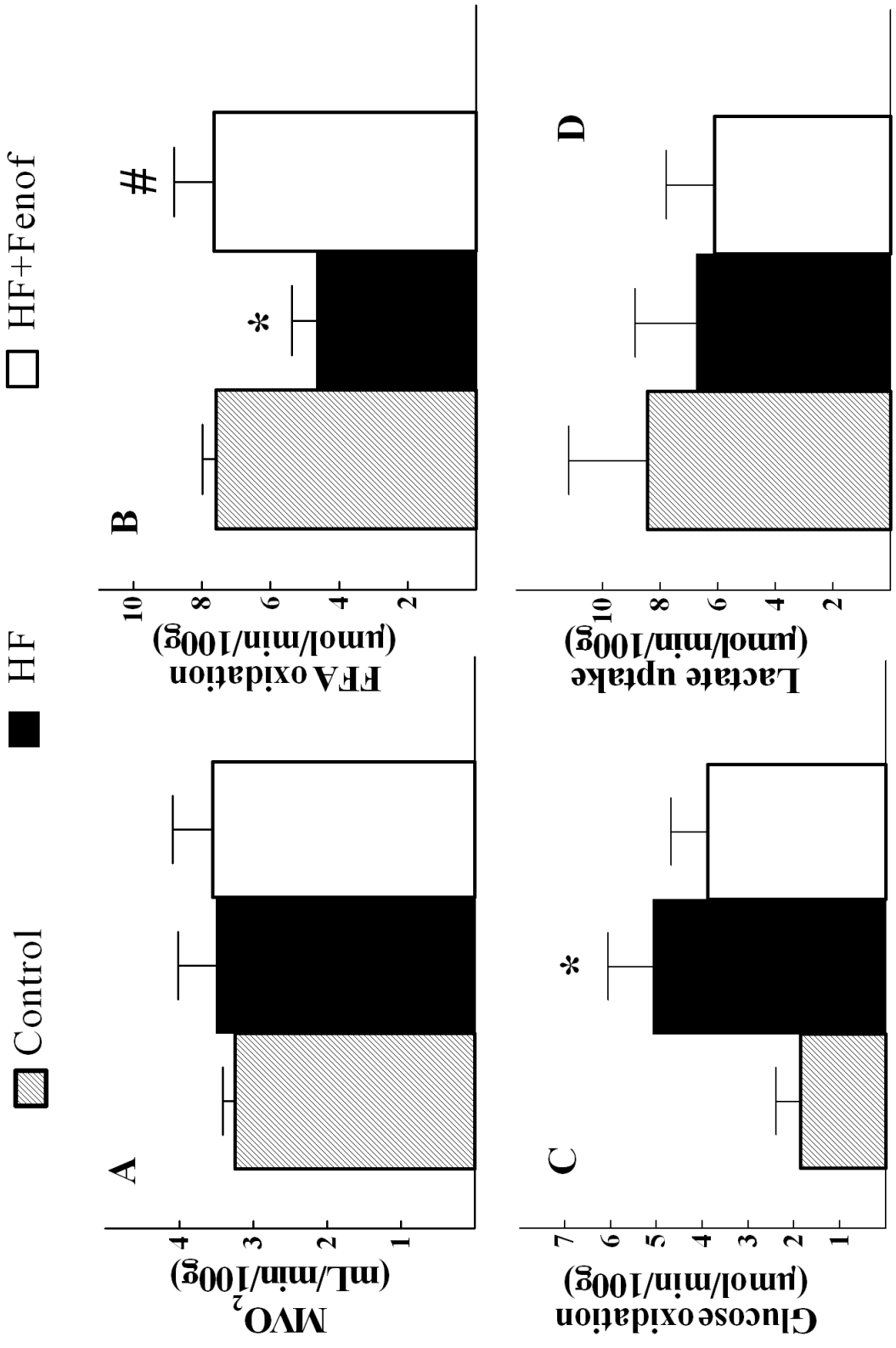

Figure 4

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Figure 5