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The PPAR- α activator fenofibrate fails to provide myocardial protection in ischemia and reperfusion in pigs

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Abstract

Rodent studies suggest that peroxisome proliferator-activated receptor- α (PPAR- α) activation reduces myocardial ischemia-reperfusion (I/R) injury and infarct size; however, effects of PPAR- α activation in large animal models of myocardial I/R are unknown. We determined whether chronic treatment with the PPAR- α activator fenofibrate affects myocardial I/R injury in pigs. Domestic farm pigs were assigned to treatment with fenofibrate 50 mg·kg⁻¹·day⁻¹ orally or no drug treatment, and either a low-fat (4% by weight) or a high-fat (20% by weight) diet. After 4 wk, 66 pigs underwent 90 min low-flow regional myocardial ischemia and 120 min reperfusion under anesthetized open-chest conditions, resulting in myocardial stunning. The high-fat group received an infusion of triglyceride emulsion and heparin during this terminal experiment to maintain elevated arterial free fatty acid (FFA) levels. An additional 21 pigs underwent 60 min no-flow ischemia and 180 min reperfusion, resulting in myocardial infarction. Plasma concentration of fenofibric acid was similar to the EC₅₀ for activation of PPAR- α in vitro and to maximal concentrations achieved in clinical use. Myocardial expression of PPAR- α mRNA was prominent but unaffected by fenofibrate treatment. Fenofibrate increased expression of carnitine palmitoyltransferase (CPT)-I mRNA in liver and decreased arterial FFA and lactate concentrations (each $P < 0.01$). However, fenofibrate did not affect myocardial CPT-I expression, substrate uptake, lipid accumulation, or contractile function during low-flow I/R in either the low- or high-fat group, nor did it affect myocardial infarct size. Despite expression of PPAR- α in porcine myocardium and effects of fenofibrate on systemic metabolism, treatment with this PPAR- α activator does not alter myocardial metabolic or contractile responses to I/R in pigs.

Keywords

nuclear receptor; fibric acid derivative; energy metabolism; cytokine; ventricular function

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors involved in regulation of energy substrate metabolism and inflammatory responses. Millions of patients are treated with fibrate PPAR- α activators for dyslipidemia, and many of these patients have, or are at risk to develop, ischemic heart disease. Although there is prominent expression of PPAR- α in myocardium (12), the function of PPAR- α in the heart remains uncertain. In particular, it is unknown whether activation of PPAR- α modifies responses to myocardial ischemia-reperfusion (I/R) injury.

Several studies in rodents have indicated that pharmacological activation of PPAR- α is protective in myocardial I/R, helping to preserve contractile function and/or reduce infarct size (6, 29, 32, 37). On the other hand, transgenic mice with cardiac-specific overexpression of PPAR- α develop a cardiomyopathy characterized by excessive myocardial neutral lipid accumulation (11, 13), and PPAR- α null mice have better functional recovery from I/R than wild-type mice, particularly when circulating free fatty acids (FFA) are elevated. (21). Finally, while compensated left ventricular (LV) hypertrophy in the mouse is associated with deactivation of PPAR- α and a switch from FFA to glucose metabolism (5), reactivation of PPAR- α with a ligand restores myocardial FFA utilization and produces severe contractile dysfunction (36). In sum, rodent studies are divided in suggesting a potential for benefit or harm from PPAR- α activation in myocardial I/R. In large animals, cardiac effects of PPAR- α activation are undefined. Moreover, there is precedent for significant species variation in PPAR-mediated responses (2, 3, 15, 19). Thus the goal of the present study was to determine the effects of PPAR- α activation in myocardial I/R injury in pigs, evaluating myocardial substrate utilization, expression of inflammatory mediators, accumulation of neutral lipid, and, most importantly, contractile function.

METHODS

Treatment groups

The experimental protocol was approved by the Institutional Animal Care and Use Committee. Eighty-seven domestic farm pigs of either sex were obtained at an average weight of 20 kg. Pigs were fed one of two diets, and in each diet group, pigs were either treated with fenofibrate or not. Sixty pigs were fed low-fat (4% by weight) laboratory pig chow (no. 5084, Purina Mills, St. Louis, MO). Of pigs fed this low-fat diet, 33 were treated with fenofibrate (Sigma, St. Louis, MO), 50 mg·kg⁻¹·day⁻¹ orally, and 27 received no treatment. Fenofibrate was mixed into the filling of fruit cookies that were fed by hand to the pigs to ensure ingestion of each dose. Untreated pigs received cookies without added fenofibrate. Twenty-seven pigs were fed a high-fat (20% by weight) diet formulated by Research Diets (New Brunswick, NJ) from Purina no. 5084 chow with added soybean and safflower oils (9.2% each by weight). Because the high-fat diet contains very little saturated fat and no added cholesterol, it is not atherogenic. Casein and soy protein (5.5% each by weight) were added to the high-fat chow to achieve protein content similar to the low-fat chow. Of the pigs fed the high-fat diet, 14 were treated with fenofibrate, 50 mg·kg⁻¹·day⁻¹, and 13 were untreated. During the fourth week of treatment with fenofibrate, venous blood specimens were withdrawn from the thoracic inlet before and ~5 h after dosing to determine plasma concentrations of fenofibric acid, the principal active metabolite of fenofibrate. The 5-h time point was chosen because peak plasma concentrations of fenofibric acid are attained ~5 h after an oral dose of fenofibrate in humans (4).

Anesthesia, surgery, and instrumentation of the heart for low-flow ischemia and reperfusion

After 4 wk of assigned diet with or without fenofibrate treatment, 66 pigs underwent a terminal experiment of low-flow myocardial ischemia and reperfusion. The methods for anesthesia, surgery, instrumentation of the heart, and physiological monitoring have been described previously (35). On the morning of the experiment, after an overnight fast, pigs were given a final dose of fenofibrate mixed in cookie filling or cookies without fenofibrate. Pigs were sedated with ketamine HCl (25 mg/kg im), anesthetized with α -chloralose (100 mg/kg iv induction, 20–30 mg·kg⁻¹·h⁻¹ iv maintenance), mechanically ventilated with oxygen-enriched air, and wrapped in a circulating warm water blanket to help maintain body temperature (25). Arterial blood glucose was monitored every 15 min, and an intravenous infusion of 10% glucose was adjusted to maintain arterial blood glucose at ~4 mmol/l.

Propranolol (1 mg/kg iv) and atropine (0.2 mg/kg iv) were administered to block autonomic reflexes that would otherwise influence measures of intrinsic myocardial function. Normal saline solution was administered at a rate of 150 ml/h iv.

Micromanometer catheters were placed in the aortic arch and LV (Fig. 1). A fluid-filled catheter was placed in the left atrium for injection of microspheres. Bipolar pacing electrodes were affixed to the left atrial appendage and used to maintain heart rate slightly greater than the spontaneous rate. An adjustable hydraulic occluder (In Vivo Metrics, Healdsburg, CA) and an ultrasonic flow probe (Transonic, Ithaca, NY) were placed around the proximal portion of the left anterior descending coronary artery (LAD) to produce and monitor the severity of regional myocardial ischemia. A catheter was placed in the anterior interventricular coronary vein to sample effluent blood from the ischemic region. An array of four ultrasonic crystals was implanted in the subendocardium of the anterior LV in the center of the ischemic region. The crystals were connected to a digital sonomicrometer (Sonometrics, London, Ontario, Canada) to measure the instantaneous regional wall area subtended by each array. In pigs that had been fed the high-fat diet, levels of FFA were elevated during the terminal experiment by infusion of triglyceride emulsion (Liposyn 10%, 50 ml/min) and heparin (15,000 IU iv initially, then 5,000 IU iv hourly).

Low-flow I/R protocol

After instrumentation of the heart, steady-state (baseline) measurements of hemodynamics, regional LV function, myocardial blood flow, arterial and coronary venous concentrations of glucose, lactate, and FFA, triglycerides, and plasma concentrations of insulin and fenofibric acid were made. Next, regional low-flow myocardial ischemia was produced by gradual constriction of the LAD using a microsyringe connected to the hydraulic occluder, while coronary flow was continuously monitored with the flowmeter. LAD flow was reduced to 50% of baseline and maintained at this level (± 1 ml/min) for 90 min by fine adjustment of the microsyringe. We and others have previously shown that this duration and severity of ischemia in pigs results in profound myocardial stunning but not infarction (17, 24). Fine adjustments of the microsyringe maintained constant LAD flow (precision 1 ml/min) throughout the ischemic period. Lidocaine (0.6 mg/kg iv) was administered before the onset of ischemia and again every 30 min during ischemia to prevent arrhythmias. At 90 min ischemia, a second set of measurements of hemodynamics, LV function, blood flow, and substrate concentrations was made. The LAD occluder was then gradually released over 2–3 min to allow reperfusion. A third set of all measurements was made at 90 min of reperfusion, and a final set of measurements of hemodynamics and LV function was made at 120 min of reperfusion. Selected 10-s intervals of hemodynamic and sonomicrometry data were digitized at a frequency of 200 Hz and analyzed using custom software. After conclusion of the in vivo protocol and euthanasia, the heart was removed and myocardial samples were quickly excised from ischemic and nonischemic regions of the LV for analysis of regional myocardial blood flow, expression of PPAR- α and carnitine palmitoyltransferase (CPT)-I mRNA, content of proinflammatory cytokines, and presence of intramyocardial lipid by Oil Red O staining. A sample of liver was excised for measurement of CPT-I expression.

Assessment of LV systolic function

Hemodynamic data were digitized at 200 Hz and analyzed using custom software built on a LabView (National Instruments) platform. LV pressure vs. regional wall area loops were recorded during brief suspension of mechanical ventilation. Regional LV contractile function was assessed by fractional systolic wall area reduction and by a regional external work index. The former is a two-dimensional analog to segment shortening in one dimension or ejection fraction in three dimensions. It was calculated as the difference

between end-diastolic and end-systolic wall area, divided by end-diastolic wall area. An index of regional external work was calculated as the area of a LV pressure vs. wall area loop inscribed in one cardiac cycle. It should be noted that these measures of contractile function are sensitive to loading conditions, which change during ischemia and reperfusion. However, these measures may be compared between groups under a given experimental condition, provided that loading conditions are similar in both groups.

Measurement of regional myocardial blood flow, substrate uptake, and fenofibric acid concentration

Under each experimental condition, regional myocardial blood flow was measured in three transmural layers with fluorescent microspheres, as previously described (26). Paired blood samples were withdrawn simultaneously from arterial and anterior interventricular coronary vein catheters for measurement of substrate concentrations. Glucose and lactate concentrations were measured using an autoanalyzer (YSI, Yellow Springs, OH). FFA concentration was measured by spectrophotometric assay (Wako Diagnostics) and triglycerides by enzymatic colorimetric assay. Regional myocardial substrate uptake was calculated as the product of transmurally averaged myocardial blood flow and the coronary arteriovenous concentration difference of the substrate of interest. Plasma insulin was measured by radioimmunoassay. Plasma concentration of fenofibric acid was measured by high-performance liquid chromatography and tandem mass spectroscopy, with a lower limit of quantitation of 0.05 µg/ml and precision (coefficient of variation) of 4% (SFBC Analytical Laboratories, North Wales, PA).

Myocardial expression of PPAR- α

Myocardial homogenates were prepared from frozen specimens of subendocardium from the non-ischemic and ischemic regions of five hearts from each of the four diet and treatment groups. Tissue samples were obtained immediately after euthanasia. For each diet group and for each myocardial region (nonischemic or I/R), samples from untreated and fenofibrate-treated pigs were compared on the same gel. Expression of PPAR- α mRNA was assessed by ribonuclease protection assay by using a riboprobe derived from the published nucleotide sequence for mouse PPAR- α (GenBank BC016892). RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (10 µg) prepared from each homogenate was hybridized overnight with the PPAR- α riboprobe at 42°C. Digestion was carried out by using 1:50 ribonuclease T₁ (Ambion, Austin, TX), and the protected fragments were separated on a 5% denaturing polyacrylamide gel. The band corresponding to PPAR- α was quantified with a phosphorimager and ImageQuant software (Amersham, Piscataway, NJ) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal control for RNA loading.

Expression of CPT-I

Probes specific for both the porcine liver (CPT-I α) and muscle (CPT-I β) genes were amplified from normal pig heart and liver with PCR-ready cDNAs (BioChain, Hayward, CA) by using 20-mer primers complementary to bases 196–215 (forward) and 390–409 (reverse) for CPT-I α and bases 26–45 (forward) and 182–201 (reverse) for CPT-I β , including *Bam*H1 and *Hind*III cloning sequences. The CPT-I α and CPT-I β sequences are GenBank AF284831 and AF284832. PCR reactions were carried out using cloned Pfu polymerase (Stratagene, La Jolla, CA), and products were gel-purified using QIAquick gel extraction (QIAGEN, Valencia, CA). After sequential digestions with *Bam*H1 and *Hind*III, probe sequences were cloned into pBSII(SK–) (Stratagene) by using T₄ DNA Ligase (Invitrogen). Antisense transcripts to both CPT-I genes and human 18S were prepared with MEGAshortscript (Ambion) with removal of unincorporated nucleotides by MEGAclear. Probes were subsequently mixed and hybridized overnight with 15 µg of RNA from

myocardium (subendocardium from I/R region) and 15 µg of RNA from liver of each experimental pig. After hybridization, samples were digested with 1:50 A:T₁ ribonuclease mix, and protected fragments were separated on a 5% denaturing polyacrylamide gel. Band intensity corrected for RNA loading was expressed as the fraction of the 18S signal.

Proinflammatory cytokines

Myocardial tissue content of three proinflammatory cytokines was measured in subendocardial samples from ischemic-reperfused and nonischemic regions by using enzyme-linked immunosorbent assay kits containing antibodies to porcine interleukin (IL)-1β and interferon (IFN)-γ (BioSource, Camarillo, CA) and IL-6 (R&D Systems, Minneapolis, MN). We previously demonstrated that cytokine content in nonischemic regions of hearts subjected to regional ischemia and reperfusion was similar to that measured in sham experiments with identical anesthesia, instrumentation, and time course, but without I/R (35).

Assessment of myocardial lipid content by Oil Red O staining

Frozen subendocardial samples from nonischemic and I/R regions of each heart were sectioned with a cryostat (12–15 µm thickness) and stained with Oil Red O for examination under 100× to 600× magnification. Each sample was given a semiquantitative score based on the following criteria: 0, no visible Oil Red O staining; 1, light patchy staining involving a minority of cells in each field; 2, moderate staining involving up to half the cells in each field; 3, intense staining involving the majority of cells in each field. The scores of three blinded observers (M. Rizeq, G. G. Schwartz, and Y. Xu) were averaged.

Effect of fenofibrate on myocardial infarct size

Twenty-one additional pigs from the low-fat diet group were treated with fenofibrate, 50 mg·kg⁻¹·day⁻¹ orally, for 4 wk with the last dose on the morning of the terminal experiment ($n = 10$), or received no treatment ($n = 11$). These pigs underwent a terminal experiment of 60-min complete occlusion of the LAD, followed by 180 min reperfusion. Amiodarone (30 mg/kg iv loading dose followed by 0.5 mg·kg⁻¹·min⁻¹ iv infusion) was administered beginning 45 min before ischemia, and lidocaine, 1 mg/kg iv, was administered before ischemia and every 30 min during ischemia and reperfusion. Ventricular fibrillation was treated by defibrillation with internal paddles. After euthanasia, myocardial infarct size was quantified as described by Downey et al. (10). The LAD was reoccluded, the ascending aorta cross-clamped, and 20 ml of 2% Evans blue solution was infused into the aortic root. The heart was excised, cooled in iced saline solution, wrapped in plastic food wrap, placed at -80°C for 30 min, and sliced from apex to base into 3-mm-thick rings. Right ventricular tissue was removed and rings were weighed, incubated in 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 20 min followed by 10% formalin for 15 min, and then photographed with a digital camera. Images were analyzed using Image J software (National Institutes of Health, Bethesda, MD). Area at risk was defined by the absence of Evans blue staining and area of infarction by the absence of TTC staining.

Statistical analysis

Data are expressed as means ± SE. Variables with one measurement per pig were analyzed by two-way ANOVA with factors fat availability and treatment assignment. Variables with more than one measurement per pig were analyzed by three-way ANOVA with factors fat availability, treatment assignment, and either experimental condition or myocardial region (repeated measures of the last factor). Main effects of each factor and key interactions between factors were examined in each model. A significance level of 0.05 was specified for all analyses.

RESULTS

Growth and development

Growth and development were similar with low- or high-fat diet and with fenofibrate treatment or no treatment. At the time of the terminal experiment, pigs fed the low-fat diet weighed 30 ± 1 kg (fenofibrate treated) and 29 ± 1 kg (untreated); pigs fed the high-fat diet weighed 31 ± 2 kg (fenofibrate treated) and 31 ± 1 kg (untreated). Whole heart weights in these four groups were 153 ± 6 , 160 ± 9 , 159 ± 9 , and 159 ± 4 g, respectively [P = not significant (NS)].

Arrhythmia

During low-flow I/R, 6 of 23 pigs (26%) in the low-fat/fenofibrate group and 3 of 16 pigs (19%) in the low-fat/untreated group developed ventricular fibrillation; one pig in each group was successfully defibrillated, completed the experimental protocol, and provided analyzable data. A total of 18 pigs in the low-fat/fenofibrate group and 14 pigs in the low-fat/untreated group completed the experimental protocol. In the high-fat/fenofibrate and high-fat/untreated groups, respectively, 3 of 14 pigs (21%) and 1 of 13 pigs (8%) suffered lethal ventricular fibrillation during low-flow I/R. A total of 11 pigs in the high-fat/fenofibrate group and 12 pigs in the high-fat/untreated group completed the low-flow I/R protocol. Of 21 pigs subjected to experimental myocardial infarction, all but one suffered at least one episode of ventricular fibrillation. Ten pigs (4 untreated and 6 fenofibrate treated) could not be defibrillated, leaving six untreated and five fenofibrate-treated pigs that completed the protocol and provided analyzable data. There was no significant difference in the incidence of ventricular fibrillation between fenofibrate-treated and untreated pigs in either diet group or in either ischemia protocol.

Expression of PPAR- α

Myocardial PPAR- α expression (normalized to GAPDH) was compared in untreated and fenofibrate-treated pigs in both low- and high-fat groups. There was prominent expression of PPAR- α in both nonischemic and I/R regions of all hearts (Fig. 2). However, PPAR- α expression was unaffected by fenofibrate treatment in either the low-fat or the high-fat group or in either myocardial region.

Plasma concentration of fenofibric acid

After 3 wk of daily treatment, peak and trough plasma concentrations of fenofibric acid were 15 ± 2 and 3 ± 1 $\mu\text{g/ml}$ (47 ± 6 and 9 ± 1 μM) in the low-fat group, and 25 ± 3 and 5 ± 1 $\mu\text{g/ml}$ (78 ± 9 and 15 ± 3 μM) in the high-fat group. Higher concentrations in the high-fat group are consistent with the enhanced absorption of fenofibrate with a fatty meal. During the terminal experiment of I/R, plasma fenofibric acid concentration averaged 7 ± 1 $\mu\text{g/ml}$ (22 ± 3 μM) in both low-fat and high-fat groups. These concentrations are similar to those achieved in human subjects treated with maximum approved dosages of fenofibrate (4) and to the EC_{50} of fenofibric acid for activation of murine or human PPAR- α in vitro (18–30 μM) (34). The demonstration of PPAR- α expression in myocardium and of pharmacologically relevant concentrations of fenofibric acid in plasma fulfills two necessary conditions for potential cardiac effects of a PPAR- α activator in this model.

Expression of CPT-I

The mRNA expression of CPT-I α (liver isoform) and CPT-I β (muscle isoform) are shown in Fig. 3. In liver, expression of CPT-I α was greater in pigs treated with fenofibrate compared with untreated pigs ($P < 0.01$). This effect of fenofibrate treatment was more prominent in the low-fat groups than in the high-fat groups (interaction $P = 0.08$). In

myocardium, expression of CPT-1 β was not affected by fenofibrate treatment in either low-fat or high-fat groups.

Circulating substrate concentrations and myocardial substrate utilization

During the fourth week of assigned diet, plasma FFA concentration was measured before and 5 h after a feeding; these two measurements did not differ significantly in any group, and data in Table 1 represent the average of the two measurements in each pig. As expected, FFA levels measured during the fourth week of assigned diet were higher ($P < 0.001$) in pigs fed the high-fat diet compared with the low-fat diet. FFA levels measured during dietary intervention tended to be lower with fenofibrate treatment; this effect approached but did not reach statistical significance ($P = 0.09$).

Table 2 shows arterial concentrations of insulin, glucose, lactate, and FFA measured during the terminal experiment under preischemic conditions, at 90 min of low-flow ischemia, and after 90 min of reperfusion. Arterial insulin and glucose concentrations were similar in all groups and under all conditions. As expected, arterial FFA concentrations were higher under high-fat conditions than under low-fat conditions ($P < 0.001$). Treatment with fenofibrate significantly reduced arterial FFA concentration ($P < 0.01$) and arterial lactate concentration ($P < 0.001$) during the terminal I/R experiment. In conjunction with the increased liver expression of CPT-1 α , these findings indicate a significant systemic metabolic effect of fenofibrate in this model. Under low-fat conditions, arterial triglyceride concentrations were below the lower limit of the assay system (0.17 mmol/l) in the majority of pigs. Under high-fat conditions, arterial triglyceride concentration was 0.5 ± 0.1 mmol/l in both the untreated and the fenofibrate-treated group.

Despite the effect of fenofibrate treatment on arterial FFA and lactate concentrations, there was no accompanying effect of fenofibrate on myocardial substrate uptake: Fig. 4 shows myocardial substrate uptake before, during, and after low-flow ischemia. As expected, high-fat conditions increased myocardial FFA uptake and reciprocally decreased myocardial glucose and lactate uptake compared with low-fat conditions. However, fenofibrate treatment had no effect on myocardial FFA, glucose, or lactate uptake under either high-fat or low-fat conditions. Myocardial triglyceride uptake could not be measured under low-fat conditions because of low circulating concentrations. Under high-fat conditions, myocardial triglyceride uptake averaged $0.03 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ in both untreated and fenofibrate-treated pigs under preischemic conditions and fell to nil in both groups during ischemia and reperfusion.

Hemodynamics, myocardial blood flow, and LV contractile function

Baseline (preischemic) hemodynamics, myocardial blood flow, and measures of regional LV systolic function were similar in each group (Table 3). Compared with pigs in the low-fat group, pigs in the high-fat group had lower mean aortic and LV systolic pressure, particularly during ischemia and reperfusion. Total LAD flow was higher and transmural anterior LV blood flow was lower in the high-fat compared with the low-fat groups; however, blood flow to the subendocardium of the ischemic region (where contractile function and cytokine expression were measured) did not differ in any group. As expected, contractile function declined during ischemia and remained depressed after reperfusion in all groups (Fig. 5). Importantly, fenofibrate treatment had no effect on any of these variables at either fat level or under any experimental condition.

Expression of proinflammatory cytokines

Figure 6 shows IL-6, IL-1 β , and IFN- γ protein concentrations in subendocardial samples from nonischemic and I/R regions of the LV. By three-way ANOVA, there was a significant

positive main effect of I/R on expression of each cytokine. The effect of I/R was evident under both low- and high-fat conditions for IL-6, but only under low-fat conditions for IL-1 β and IFN- γ . Fenofibrate treatment had a modest but significant negative main effect on expression of IL-1 β ($P < 0.05$) but no main effect on expression of IL-6 or IFN- γ .

Intramyocardial neutral lipid

Representative photomicrographs and group semiquantitative scores of Oil Red O staining in subendocardial samples are shown in Fig. 7. By three-way ANOVA, there was a significant positive main effect of high-fat conditions ($P < 0.001$). There was also a significant interaction ($P < 0.05$) between myocardial region and fat availability, such that lipid accumulation was increased by I/R under high-fat but not low-fat conditions. However, there was no effect of fenofibrate treatment on myocardial lipid accumulation at either fat level or in either myocardial region.

Myocardial infarct size

As shown in Table 4, there was no significant difference between groups in area at risk and no significant effect of fenofibrate treatment on infarct size.

DISCUSSION

In a model of I/R in the in situ porcine heart, 1 mo of pretreatment with the PPAR- α activator fenofibrate had no effect on myocardial substrate utilization, lipid accumulation, contractile function, or infarct size. Effects of treatment were absent even under conditions of increased FFA availability. A protective effect of fenofibrate was absent despite fulfillment of three necessary conditions for such an effect: expression of PPAR- α in porcine myocardium was prominent; concentrations of fenofibric acid in plasma were comparable to the EC₅₀ for activation of PPAR- α in vitro (34) and to concentrations achieved in effective clinical use of fenofibrate (4); and biological activity of fenofibrate in this model was evident from a significant increase in liver expression of CPT-1 α , a decrease in arterial FFA and lactate concentrations during the terminal I/R experiment, and a decrease in myocardial IL-1 β expression.

We chose to use fenofibrate as the test compound in these experiments because it is used widely in clinical practice, because its active metabolite fenofibrate distributes well to myocardium (33), and because established methods exist to measure fenofibric acid concentration in plasma. Among the fibric acid derivatives in current clinical use, fenofibrate has the highest affinity and is the most selective for PPAR- α . Synthetic ligands with higher affinity for PPAR- α than fenofibrate have been developed (34) but are untested in large animals or humans and have no established methods for measurement of plasma concentrations.

The neutral findings of the present study stand in contrast to the protective effects of PPAR- α activation demonstrated in several prior studies of myocardial I/R in rodents. Wayman et al. (32) showed that acute treatment with clofibrate or WY-14643 (0.3–1.0 mg/kg), administered 30 min before 25-min occlusion of the anterior descending coronary artery and 2 h of reperfusion in rats, reduced infarct size by ~40%. Bulhak et al. (6) reported similar findings using a similar model. Taberno et al. (29) subjected mice to 30 min global no-flow ischemia and 1-h reperfusion. Ten days of pretreatment with fenofibrate (15 mg/day) reduced infarct size to ~5% of LV mass compared with ~20% of LV mass in untreated mice. There was also a beneficial effect of fenofibrate on recovery of contractile function. Moreover, these investigators found no protective effect of fenofibrate in PPAR- α null mice, indicating that the protective effects in wild-type mice are likely mediated through

PPAR- α . Similarly, Yue et al. (37) found that 2 days of pretreatment with the PPAR- α activator GW-7647 reduced myocardial infarct size and postischemic contractile dysfunction in wild-type mice. Protection by GW-7647 was associated with inhibition of nuclear factor (NF)- κ B in the heart and decreased expression of proinflammatory cytokines but was not evident in PPAR- α null mice. Other studies have found protective effects of PPAR- α activation in splanchnic (8) and renal (27) I/R injury in rats. In the former case, protection was associated with reduced expression of proinflammatory cytokines in the gut. In sum, these studies indicate that pharmacological activation of PPAR- α significantly attenuates I/R injury in rodents.

On the other side of the coin, studies in transgenic mice with cardiac-specific overexpression of PPAR- α suggest that excessive signaling through this pathway may be deleterious. These mice not only demonstrate increased myocardial expression of proteins involved in fatty acid uptake and oxidation but also exhibit a pathological phenotype characterized by myocardial lipid accumulation, biventricular hypertrophy, and contractile dysfunction (13). The severity of cardiomyopathy was exacerbated by feeding a high-fat diet (11). In contrast, we found that exposure of pigs to a PPAR- α activator caused no impairment of cardiac function and no increase in myocardial lipid content, even under conditions of increased FFA availability.

The absence of either protective or deleterious effects of fenofibrate in the present study may reflect species differences in ligand activation and effects of PPAR- α . The precedent for such differences is well established in tissues other than myocardium and in species other than pig (2, 7). For example, fibrates are more effective than linoleic acid in promoting PPAR- α activation in cultured rat hepatoma cells, whereas linoleic acid is more effective than fibrates in human hepatoma cells (2). Fenofibrate increases the expression of fatty acyl-CoA oxidase in rat hepatocytes but not human hepatocytes (16). Similarly, peroxisome proliferation in response to activation of PPAR- α is observed in rat but not human hepatocytes (3, 7). Fibrates decrease hepatic expression of apolipoprotein A-I in rodents but increase expression in humans (30). These differences appear to be independent of the level of PPAR- α expression (16, 19) and rather may reflect species differences in the ligand-binding domain of the receptor (15). Analogous to the findings in the present study, PPAR- γ activation with thiazolidinedione compounds is not protective in myocardial I/R in pigs (35) but appears to be protective in rodents (32, 38).

The absence of observed effects of fenofibrate on myocardial substrate uptake in the current experiments is consistent with the fact that we did not observe an effect of treatment on myocardial CPT-I expression. Taken in the context of the significant effects of fenofibrate on liver CPT-I expression and plasma FFA and lactate concentrations, our findings indicate significant tissue specificity of response to PPAR- α activation in the pig. Our findings are consistent with a study showing that mice pretreated with a PPAR- α agonist for 8 wk did not differ from untreated mice in terms of myocardial glucose or FFA oxidation or myocardial expression of PPAR- α or PPAR- α target genes (1). However, our findings contrast with observations in cultured neonatal rat cardiomyocytes, in which exposure to PPAR- α ligands increases FFA oxidation along with the expression of genes involved in fatty acid transport and oxidation (14).

Anti-inflammatory effects of PPAR- α are thought to be mediated through negative regulation of the transcription factors NF- κ B and activator protein-1, resulting in decreased expression of their target genes, particularly IL-6 (9, 22, 28). However, we observed no effect of fenofibrate treatment on myocardial IL-6 expression. Fibrates have also been reported to reduce levels of IL-1 β in plasma (31) and to attenuate expression of IFN- γ by T-lymphocytes (18, 20). We did observe a quantitatively modest albeit statistically significant

attenuation of myocardial IL-1 β expression with fenofibrate. The physiological importance of this effect is uncertain. We also observed that myocardial IL-1 β and IFN- γ did not increase with I/R under high-fat conditions. This may reflect an anti-inflammatory effect of the n-6 polyunsaturated fatty acids (23), which were a large component of the high-fat diet enriched in soybean and safflower oils.

A limitation of this study is that we tested only one dose of fenofibrate because of the large number of pigs required for the experiments. We cannot exclude the possibility that use of a higher dose of fenofibrate or another PPAR- α activator would have produced different effects. However, we believed that it was most important to evaluate an agent already in wide clinical use at a dose that results in clinically relevant plasma concentrations of the active metabolite.

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Fig. 1.
Instrumentation of the heart. LV, left ventricle; LA, left atrium.

Fig. 2.

Myocardial expression of peroxisome proliferator-activated receptor (PPAR)- α mRNA by ribonuclease protection assay. *A*: representative lanes from ribonuclease protection assays demonstrating myocardial expression of PPAR- α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. From *left to right*, the 4 panels show examples from low-fat availability/non-ischemic region, low-fat availability/ischemic-reperfused (Isc/Rep) region, high-fat availability/nonischemic region, and high-fat availability/ischemic-reperfused region. Each panel shows results from 2 untreated and 2 fenofibrate-treated hearts. *B*: group data, expressed as means $\pm$ SE with 11–18 samples in each category. In each gel, the PPAR- α /GAPDH ratio in each lane was normalized to the mean ratio of the samples from untreated hearts in that gel. Fenofibrate treatment had no effect on PPAR- α mRNA expression under conditions of either low or high fat availability or in either myocardial region.

Fig. 3. Liver and myocardial expression of carnitine palmitoyltransferase-I (CPT-I) mRNA. *Left:* expression of CPT-1α (liver isoform) in porcine liver. *Right:* expression of CPT-1β (muscle isoform) in porcine subendocardial myocardium. Measurements are means $\pm$ SE with $n = 12$ in each category. Two-way ANOVA with factors fat availability and treatment showed that fenofibrate treatment had a significant positive main effect on expression of CPT-1α in liver (* $P < 0.01$) but had no significant effect on expression of CPT-1β in myocardium.

Fig. 4. Myocardial substrate uptake. Data are from a terminal low-flow ischemia-reperfusion experiment that followed 4 wk of feeding either a low-fat or a high-fat diet (see text). Uptake of free fatty acids (FFA), glucose, and lactate in the ischemic-reperfused region of the left ventricle are plotted as means $\pm$ SE, with 11–19 pigs in each group. Pre-isc, baseline before ischemia; Isc, at 90 min ischemia; Rep, at 90 min of reperfusion. In the groups fed a high-fat diet, arterial FFA concentrations were increased in the terminal experiment by infusion of triglyceride emulsion and heparin. In 3-way ANOVA with factors fat availability, fenofibrate treatment, and experimental condition, high-fat conditions increased

myocardial FFA uptake and decreased glucose and lactate uptake (* $P < 0.01$). However, there was no significant effect of fenofibrate treatment on uptake of any substrate.

Fig. 5.

Regional LV systolic function (regional external work). Regional external work of the ischemic-reperfused region of the left ventricle is plotted as a fraction of baseline (preischemic) values. The absolute values of regional external work were similar at baseline in all 4 groups (see Table 2). Data are means $\pm$ SE with 11–19 pigs in each group. Pre-isc, preischemic baseline; Isc, at 90 min ischemia; Rep, at 90–120 min reperfusion. In all groups, regional external work declined during ischemia and declined further during reperfusion. However, there were no significant differences in regional external work among groups.

Fig. 6.

Proinflammatory myocardial cytokine expression. Protein expression by enzyme-linked immunosorbent assay expressed as ng/g protein content. *Top*: interleukin (IL)-6. *Middle*: IL-1 β . *Bottom*: interferon (IFN)- γ . Data are means $\pm$ SE of 10–14 pigs in each group. In 3-way ANOVA models with factors fenofibrate treatment, fat availability, and myocardial region, there was a significant negative main effect of fenofibrate treatment on IL-1 β (* $P < 0.05$) but not on IL-6 or IFN- γ . non-isc, Nonischemic; rep, reperfused; LF, low fat availability; HF, high fat availability.

Fig. 7.

Intramyocardial neutral lipid accumulation assessed by Oil Red O staining: *Top*: representative photomicrographs (600 $\times$) showing pattern of Oil Red O staining in previously frozen subendocardial samples from ischemic-reperfused (I/R) region of hearts from pigs administered 4 wk of low-fat or high-fat diet, with or without fenofibrate treatment. Pigs in the high-fat groups received an infusion of triglyceride emulsion and heparin during the terminal I/R experiment to maintain elevated fat availability. In the specimens from the low-fat groups, there is patchy staining with Oil Red O involving a small minority of myocytes. In the specimens from the high-fat groups, there is more pronounced staining involving approximately half of the myocytes. *Bottom*: semiquantitative histologic score of Oil Red O staining in subendocardial samples. A score of 0 was assigned for no staining, 1 for light patchy staining involving a minority of cells in each field, 2 for moderate staining involving up to half the cells in each field, or 3 for intense staining involving the majority of cells in each field. Data are means $\pm$ SE with 11–19 pigs in each group. U, untreated; F, fenofibrate-treated. In 3-way ANOVA with factors fat availability, fenofibrate treatment, and

myocardial region, there was a significant positive main effect of high fat (* $P < 0.001$) but no significant effect of fenofibrate treatment on Oil Red O score.

Table 1

Plasma FFA concentration during chronic feeding of low-fat or high-fat diet

	Untreated	Fenofibrate	<i>P</i> Value (Low-Fat vs. High-Fat Diet)
Low-fat diet	0.14±0.02	0.10±0.01	<0.001
High-fat diet	0.27±0.03	0.23±0.02	
<i>P</i> value (untreated vs. fenofibrate)	NS		

Values are means ± SE of free fatty acid (FFA) concentrations in mmol/l. Low-fat diet group: $n = 10$ (untreated), $n = 20$ (fenofibrate). High-fat diet group: $n = 14$ (untreated), $n = 13$ (fenofibrate). FFA concentrations were measured in venous blood before and 5 h after feeding; these 2 measurements did not differ significantly in any group, and therefore data represent the average of the 2 measurements in each pig. *P* values are for main effects determined by 2-way ANOVA. NS, not significant.

Table 2

Arterial substrate concentrations during terminal experiment

	Plasma Insulin, $\mu\text{U/ml}$	Arterial Glucose, mmol/l	Arterial Lactate, mmol/l	Arterial FFA, mmol/l
Preischemia				
LF-untreated	3 $\pm$ 0	4.0 $\pm$ 0.1	0.7 $\pm$ 0.0	0.11 $\pm$ 0.01
LF-fenofibrate	5 $\pm$ 2	4.0 $\pm$ 0.1	0.6 $\pm$ 0.0	0.09 $\pm$ 0.01
HF-untreated	4 $\pm$ 1	4.3 $\pm$ 0.2	0.7 $\pm$ 0.1	0.63 $\pm$ 0.08
HF-fenofibrate	3 $\pm$ 1	4.2 $\pm$ 0.1	0.5 $\pm$ 0.1	0.48 $\pm$ 0.04
Ischemia				
LF-untreated	3 $\pm$ 0	4.1 $\pm$ 0.1	0.8 $\pm$ 0.1	0.05 $\pm$ 0.01
LF-fenofibrate	5 $\pm$ 1	4.1 $\pm$ 0.1	0.7 $\pm$ 0.0	0.04 $\pm$ 0.00
HF-untreated	5 $\pm$ 1	4.2 $\pm$ 0.1	1.0 $\pm$ 0.1	0.54 $\pm$ 0.03
HF-fenofibrate	5 $\pm$ 1	4.2 $\pm$ 0.1	0.6 $\pm$ 0.1	0.49 $\pm$ 0.06
Reperfusion				
LF-untreated	4 $\pm$ 1	4.0 $\pm$ 0.1	0.8 $\pm$ 0.0	0.05 $\pm$ 0.01
LF-fenofibrate	5 $\pm$ 2	4.0 $\pm$ 0.1	0.7 $\pm$ 0.0	0.03 $\pm$ 0.00
HF-untreated	5 $\pm$ 1	4.2 $\pm$ 0.2	1.2 $\pm$ 0.2	0.59 $\pm$ 0.06
HF-fenofibrate	5 $\pm$ 2	4.1 $\pm$ 0.2	0.6 $\pm$ 0.1	0.43 $\pm$ 0.05
3-way ANOVA model (main effects <i>P</i> values)				
Fenofibrate treatment	NS	NS	<0.001	<0.01
Fat availability	NS	NS	NS	<0.001
Experimental condition	NS	NS	<0.001	<0.01

Values are means $\pm$ SE with 11–19 pigs in each group. LF, low fat availability; HF, high fat availability; Ischemia, measurements at 90 min of ischemia; Reperfusion, measurements at 90 min of reperfusion. SEs of zero reflect rounding.

Table 3

Hemodynamics and myocardial blood flow

	Heart Rate, beats/min	Mean Aortic Pressure, mmHg	LV Systolic Pressure, mmHg	LV End-Diastolic Pressure, mmHg	LAD Coronary Flow, ml/min	Anterior LV Transmural Blood Flow, ml·g ⁻¹ ·min ⁻¹	Anterior LV subendocardial Blood Flow, ml·g ⁻¹ ·min ⁻¹	Anterior LV Systolic Wall Area Reduction, %	Regional External Work Index, mmHg·cm ²
Preischemia									
LF-untreated	126±1	92±3	102±3	8±1	32±3	0.97±.06	0.76±.06	32±1	23±1
LF-fenofibrate	126±1	94±3	105±3	7±1	30±2	1.03±.05	0.80±.06	33±1	24±1
HF-untreated	128±2	96±4	107±4	7±1	37±3	0.94±.10	0.85±.13	32±1	23±1
HF-fenofibrate	127±2	87±5	98±4	7±2	41±3	0.95±.10	0.77±.10	28±3	19±2
Ischemia									
LF-untreated	127±1	86±3	97±3	10±1	16±2	0.48±.04	0.17±.02	3±2	7±1
LF-fenofibrate	126±1	83±2	91±3	9±1	16±1	0.45±.04	0.17±.03	6±2	9±1
HF-untreated	128±1	81±5	90±4	8±1	19±2	0.36±.06	0.16±.04	2±2	6±1
HF-fenofibrate	130±1	76±6	88±3	7±2	19±2	0.34±.05	0.15±.03	1±2	6±1
Reperfusion									
LF-untreated	128±1	86±4	95±3	10±1	28±3	0.79±.11	0.69±.11	5±2	4±1
LF-fenofibrate	126±1	80±4	92±4	10±1	24±1	0.78±.07	0.62±.09	4±1	6±1
HF-untreated	132±3	64±6	77±5	7±1	34±4	0.57±.10	0.42±.08	5±2	3±1
HF-fenofibrate	131±2	66±5	77±5	9±3	28±4	0.61±.05	0.49±.08	3±2	3±1
3-way ANOVA model (main effects <i>P</i> values)									
Fenofibrate treatment	NS	NS	NS	NS	NS	NS	NS	NS	NS
Fat availability	NS	<0.05	<0.05	NS	<0.01	<0.01	NS	NS	<0.05
Experimental condition	NS	<0.001	<0.001	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001

Values are means ± SE with 11–19 pigs in each group. Ischemia, measured at 90-min ischemia; Reperfusion, measured during the final 30 min of 120-min reperfusion; LV, left ventricle; LAD, left anterior descending coronary artery.

Table 4

Myocardial infarct size

Group	Area at Risk (fraction of LV mass)	Infarct Size (fraction of area at risk)
Untreated (<i>n</i> = 6)	0.28±0.03	0.35±0.10
Fenofibrate (<i>n</i> = 5)	0.32±0.08	0.28±0.10
<i>P</i> value	NS	NS

Values are means ± SE. Pigs treated with fenofibrate (50 mg/kg day orally) for 4 wk and untreated pigs underwent 60 min complete occlusion of the LAD followed by 3 h reperfusion. Area at risk and infarct size were determined by Evans blue and triphenyltetrazolium chloride staining, respectively.