Mice With Cardiac Overexpression of Peroxisome Proliferator–Activated Receptor γ Have Impaired Repolarization and Spontaneous Fatal Ventricular Arrhythmias

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Background—Diabetes mellitus and obesity, which confer an increased risk of sudden cardiac death, are associated with cardiomyocyte lipid accumulation and altered cardiac electric properties, manifested by prolongation of the QRS duration and QT interval. It is difficult to distinguish the contribution of cardiomyocyte lipid accumulation from the contribution of global metabolic defects to the increased incidence of sudden death and electric abnormalities.

Methods and Results—In order to study the effects of metabolic abnormalities on arrhythmias without the complex systemic effects of diabetes mellitus and obesity, we studied transgenic mice with cardiac-specific overexpression of peroxisome proliferator–activated receptor γ 1 (PPARγ1) via the cardiac α-myosin heavy-chain promoter. The PPARγ transgenic mice develop abnormal accumulation of intracellular lipids and die as young adults before any significant reduction in systolic function. Using implantable ECG telemeters, we found that these mice have prolongation of the QRS and QT intervals and spontaneous ventricular arrhythmias, including polymorphic ventricular tachycardia and ventricular fibrillation. Isolated cardiomyocytes demonstrated prolonged action potential duration caused by reduced expression and function of the potassium channels responsible for repolarization. Short-term exposure to pioglitazone, a PPARγ agonist, had no effect on mortality or rhythm in WT mice but further exacerbated the arrhythmic phenotype and increased the mortality in the PPARγ transgenic mice.

Conclusions—Our findings support an important link between PPARγ activation, cardiomyocyte lipid accumulation, ion channel remodeling, and increased cardiac mortality. (Circulation. 2011;124:2812-2821.)

Key Words: arrhythmia ■ metabolism ■ ion channels ■ animal model ■ diabetes mellitus

Diseases that affect cardiac energy metabolism and increase cardiomyocyte lipid stores, such as diabetes and obesity, are frequently associated with altered mechanical and electric function in the heart, a syndrome termed lipotoxic cardiomyopathy.1–3 After adjusting for other cardiovascular risk factors, both diabetes and obesity confer an increased risk of sudden cardiac death.4–8 and are associated with altered cardiomyocyte electric properties, manifested by prolongation of the QRS and QT intervals.9–11 The molecular mechanisms responsible for alterations in the electric properties of cardiomyocytes and the increased incidence of sudden cardiac death have not been well elucidated. An essential question is whether the diabetes mellitus–induced cardiomyocyte lipid accumulation or the diabetes mellitus–induced global metabolic defects cause the increased incidence of sudden death.

Clinical Perspective on p 2821

In contrast to diabetic patients, several models of cardiac lipid accumulation have not shown increased mortality. Transgenic (TG) mice with cardiac-restricted overexpression of the peroxisome proliferation–activated receptor α (PPARα) exhibit a cardiac metabolic phenotype that is similar to that of the diabetic heart: increased fatty acid utilization and decreased uptake and oxidation of glucose.12 These mice, which develop cardiomyocyte lipid accumulation,
demonstrate reduced potassium (K\(^+\)) channel repolarizing currents. In contrast to diabetic patients, however, TG-PPAR\(\gamma\) mice do not have significant prolongation of the cardiac action potential duration (APD) and do not have increased incidence of sudden death.\(^{13}\) Mice with cardiac-restricted overexpression of the fatty-acid transport protein 1 (FATP1), which develop cardiomyocyte lipid accumulation, demonstrate prolongation of the QTc interval because of a reduction in repolarizing voltage-gated K\(^+\) currents.\(^{14}\) These mice only have increased mortality when pregnant.

Peroxisome proliferator–activated receptor \(\gamma\) (PPAR\(\gamma\)), a transcription factor that causes lipid accumulation, insulin sensitivity, and reduced inflammation in the vessel wall,\(^{15,16}\) is typically expressed at relatively low levels in the heart. PPAR\(\gamma\) suppresses cardiac growth and embryonic gene expression.\(^{17}\) PPAR\(\gamma\) is expressed at higher levels in the human heart, especially in humans with metabolic syndrome, than in the murine heart.\(^{18,19}\) PPAR\(\gamma\) is activated by rosiglitazone and pioglitazone, drugs that are associated with heart failure and, in the case of rosiglitazone, greater cardiac mortality.\(^{20}\) Mice with cardiac-specific overexpression of PPAR\(\gamma\) have abnormal accumulation of intracellular lipids in cardiomyocytes, gradually develop dilated cardiomyopathy, and die suddenly in young adulthood, often before a reduction in systolic function; this premature demise is exacerbated by treatment with rosiglitazone.\(^{18}\) We found that these mice have spontaneous ventricular tachyarrhythmias causing sudden death, secondary to electric remodeling. Although short-term exposure to pioglitazone, a more commonly used PPAR\(\gamma\) agonist, had no effect on mortality and spontaneous ventricular arrhythmias in wild-type (WT) mice, pioglitazone further exacerbated the arrhythmic phenotype and increased the mortality in the TG-PPAR\(\gamma\) mice. Our findings support an important link among cardiac PPAR\(\gamma\) activation, cardiomyocyte lipid accumulation, and cardiac electrophysiological remodeling and describe a model for the greater incidence of sudden death in patients with diabetes mellitus.

Methods
A detailed description of methods and reagents used is provided in the online-only Data Supplement.

Telemetry, ECG Analysis, and Monophasic Action Potential Recordings
Telemetry devices (Data Sciences International, model EA-F20) were implanted in 10-week-old mice. Recordings were begun 1 week after implantation. Intervals were measured manually using Pnomah 3 software. For in vivo monophasic action potential (MAP) recordings, under general anesthesia, a thoracotomy was made between the ribs of the left side of the thorax, and a 0.25-mm-tip electrode was pressed lightly against the anterior surface of the left ventricle (LV). The ground electrode was pressed against the inner surface of the rib cage. Signals were amplified and filtered as described.\(^{21}\)

Echocardiography
Transtracheal echocardiography was performed on isoflurane-anesthetized mice using a high-resolution imaging system with a 30-MHz imaging transducer (Vevo 770; VisualSonics).\(^{18}\)

Isolation of Cardiomyocytes and Cellular Electrophysiology
Cardiomyocytes were isolated using methods previously described.\(^{22}\) Membrane currents, from noncontracting rod-shaped cells with clear striations, were measured by the whole-cell patch-clamp method\(^{23}\) using a MultiClamp 700B amplifier (Axon Instruments, Union City, CA). Solutions and voltage clamp methodologies are further described in the online-only Data Supplement.

Optical Mapping
High-resolution optical mapping experiments were performed on 16-week old TG-PPAR\(\gamma\) and WT littermate control mice as previously described.\(^{24–26}\) Briefly, hearts were isolated and perfused by the Langendorff method with warm (37°C) oxygenated Tyrode solution. After stabilization, the heart was stained with the voltage-sensitive dye Di-4-ANEPPs (8 \(\mu\)L of 2-mmol/L stock solution dissolved in DMSO), and contraction was inhibited with bledastatin (5 \(\mu\)mol/L). The heart was stimulated with a platinum electrode at 100-ms intervals.

Real-Time Polymerase Chain Reaction
Samples of ventricular tissue from 10 to 12 week old PPAR\(\gamma\) and WT littermate mice were used for real-time polymerase chain reaction. Real-time polymerase chain reaction was performed using an Applied Biosystems StepOne Plus real-time polymerase chain reaction system and inventoried primers (Applied Biosystems). Polymerase chain reactions were performed, in duplicate, for 40 cycles with automated detection of crossing threshold.

Immunoblots
The preparation and immunoblotting of heart homogenates was performed as described.\(^{27}\) Chemiluminescence signal was obtained using a Kodak Image Station and signal intensities quantified using ImageJ software.

Immunohistochemistry
Heart tissue was fixed with 4% paraformaldehyde, embedded in paraffin wax, and then sectioned. Sections were incubated with anti-Cx43 (1:200) or nonimmune rabbit polyclonal immunoglobulin G at 4°C overnight. For DAB staining, sections were exposed sequentially to 0.3% H\(_2\)O\(_2\), antirabbit swine antibody conjugated to biotin (1:500, DakoCytomation) for 1 hour, peroxidase-labeled ABC (VECTASTAIN ABC Kit, Vector Laboratories), and finally developed with DAB solution (ImmPACT DAB Peroxidase Substrate, Vector Laboratories). Sections were counterstained with hematoxylin. For immunofluorescent staining, after reaction with antirabbit donkey antibody conjugated to Alexa Fluor 488 (1:500, Invitrogen), sections were counterstained with DAPI.

Statistical Analysis
Results are presented as mean\pm SEM. The nonparametric Mann-Whitney U test was used for comparisons with n <10, and the unpaired \(t\) test with equal variances was used for comparisons of larger groups. A 2-tailed value of \(P<0.05\) was considered statistically significant, except for ECG intervals and premature ventricular complexes (PVCs) burden where the Bonferroni correction was used for multiple comparisons. Linear regression analysis was performed using GraphPad Software.

Results
Two lines of TG-PPAR\(\gamma\)1 mice have been developed and reported,\(^{18}\) showing similar phenotypes, albeit with different severities. The high-expressing line (alpha-myosin heavy-chain promoter [MHC] PPAR\(\gamma\)1H) demonstrated a reduced ejection fraction by 4 months of age. The MHC-PPAR\(\gamma\)1H mice had significantly reduced lifespan, with 50% mortality at 3 months of age before the clinical development of heart
failure. The MHC-PPARγ1H mouse line was used for all experiments.

**Young Adult PPARγ Mice Have Normal Echocardiograms and Histology**

We performed echocardiography of 10 to 12 week old TG-PPARγ and WT littermate mice. Left ventricular systolic dimension and fractional shortening were within normal limits for PPARγ mice at this age (Figure 1A–C). Histological examination did not demonstrate increased fibrosis (Figure 1D). These results suggest that the increased mortality is not related to systolic dysfunction or fibrosis. Gross structural analysis revealed that the thickness and fiber direction across the ventricular walls did not change significantly in the 10- to 12-week-old PPARγ mice compared with WT littermate controls (Figure 1D). The PPARγ mice have mild cardiac hypertrophy because the heart weight/body weight ratio is modestly increased, 13% above WT (online-only Data Supplement Figure I), consistent with prior reports from PPARγ agonist–treated mice.

**PPARγ Mice Have Fatal Ventricular Arrhythmias**

We hypothesized that an arrhythmia was the most likely cause of the sudden death and implanted telemetry to monitor the heart rhythm of 10-week-old TG-PPARγ and WT littermate mice. Echocardiograms, performed before telemeter implantation, demonstrated normal LV size and function. The average daily heart rate of the TG-PPARγ mice compared with age-matched littermate mice was similar to WT mice at 12 and 16 weeks of age (Figure 2A). The QRS duration was significantly prolonged for 16-week-old TG-PPARγ mice compared with age-matched WT littermates (Figure 2B and 2C). The QT interval was significantly prolonged at 12 and 16 weeks in TG-PPARγ mice compared with WT (Figure 2B and 2D).

The TG-PPARγ mice had significantly increased ventricular arrhythmias compared with WT at 12 and 16 weeks of age. Premature ventricular complexes were frequent in the PPARγ mice, averaging 7.4 PVCs/h in 12-week-old mice compared with <0.2 PVCs/h (≈5 PVCs/d) in age-matched WT mice (Figure 2E). Complex ectopy, such as paired PVCs or nonsustained ventricular tachycardia, occurred frequently in TG-PPARγ mice (0.08/h at 12 weeks, 1.2/h at 16 weeks) and was never observed in WT littermates. Sustained ventricular tachycardia was the cause of death in 2 of 3 TG-PPARγ mice undergoing long-term monitoring; the third mouse died from bradycardia. Death due to bradycardia is common in TG mouse models of heart disease whereas death from spontaneous ventricular tachycardia is unusual.

**Prolonged APD and Decreased K+ Current Density in PPARγ Mice**

The prolongation of the QT interval may be due to abnormalities in cardiomyocyte depolarization and/or repolarization. Monophasic action potentials, which are extracellular waveforms, were used to quantify ventricular repolarization. We measured the MAPs in WT and TG-PPARγ mice through a small thoracotomy by placing an electrode on the anterolateral surface of the beating heart during normal sinus rhythm. The APD measurements in WT mice are similar to the in vitro findings previously reported for murine heart. We found that the APD20 (PPARγ: 5.2±0.7 ms; WT 2.4±0.3 ms; P<0.01) and APD50 (PPARγ: 10.7±1.0 ms; WT 5.9±0.4 ms; P<0.01) were significantly prolonged in 12-week-old TG-PPARγ mice compared with age-matched littermates (Figure 3A and 3B). The APD90 was not significantly different in TG-PPARγ and WT mice, P=0.44. In the TG-PPARγ mice, the longer phase 2 of the action potential likely causes increased activation of K+ currents in phase 3 of the action potential, enabling a compensatory faster phase 3 repolarization. The prolongation of early repolarization (APD20 and APD50), without significant prolongation of the APD90, can be arrhythmogenic. The action potential waveforms, measured using the perforated patch clamp technique at 35°C and stimulating at 1000-ms intervals, were prolonged in the ventricular myocytes from 10- to 12-week-old TG-PPARγ mice compared with age-matched WT littermate controls (Figure 3C). Consistent with the in vivo MAP recordings, the APD20 and APD50 of the TG-PPARγ mice were prolonged compared with WT, and the APD90 was similar in the two genotypes. We conclude that K+ currents, which are important for normal ventricular repolarization, are increased in the TG-PPARγ mice.
APD\textsubscript{90} in the TG-PPAR\textgamma{} cardiomyocytes were significantly increased compared with control cardiomyocytes ($P<0.001$; Figure 3D). The APD\textsubscript{90} in the TG-PPAR\textgamma{} cardiomyocytes was also significantly increased compared with control cardiomyocytes ($P<0.001$; Figure 3B). The difference in rate between the MAP recordings and patch-clamp recording may explain the difference in APD\textsubscript{90} estimates because rate can modify repolarization currents and calcium handling.

The repolarization phase of the cardiac action potential is dependent on a balance between inward depolarizing Ca\textsuperscript{2+} and Na\textsuperscript{+} currents and outward repolarizing K\textsuperscript{+} currents. Whole-cell currents were recorded at room temperature from 10- to 12-week-old TG-PPAR\textgamma{} mice and corresponding age-matched littermate controls. Voltage-dependent Na\textsuperscript{+} and Ca\textsuperscript{2+} current densities and current voltage (I-V) relationship were similar in the TG-PPAR\textgamma{} and WT mice (Figure 4A and 4B). Boltzmann fits of the activation and inactivation revealed no changes in Na\textsuperscript{+} and Ca\textsuperscript{2+} channels' $V_{50}$ for activation and for inactivation. A late persistent Na\textsuperscript{+} current was not observed in the TG-PPAR\textgamma{} mice (Figure 4A, insets).

The voltage-dependent K\textsuperscript{+} currents, in contrast, were significantly altered in the PPAR\textgamma{} mice compared with the WT mice (Figure 4C). In mice, at least 4 distinct voltage-dependent K\textsuperscript{+} currents have been identified.\textsuperscript{32,33} Peak outward K\textsuperscript{+} current ($I_{K,peak}$) was reduced in the PPAR\textgamma{} mice ($44.2\pm4.2$ picoamps/picofarads [pA/pF]) compared with WT
mice (60.4±5 pA/pF; P=0.02). The decay phases of the voltage-dependent K\(^+\) currents in adult mouse cardiomyocytes may be fit by the sum of 2 exponentials, which denotes the fast transient K\(^+\) current I\(_{\text{K,trans}}\), a rapidly activating, very slowly inactivating current, I\(_{\text{K,slow}}\), and a nonactivating current, I\(_{\text{K,act}}\). Analysis of the decay phases demonstrated a statistically significant reduction in the current density of I\(_{\text{K,slow}}\) (WT: 23.8±2.1 pA/pF; PPAR\(_{\gamma}\) : 14.1±1.3 pA/pF; P<0.001), and a nonstatistically significant reduction in current densities of I\(_{\text{K,act}}\) (WT: 22.4±2.8 pA/pF; PPAR\(_{\gamma}\) : 16.2±3 pA/pF; P=0.15; Figure 4C). There was no significant change in the current density of I\(_{\text{K,trans}}\) (WT: 10.8±0.8 pA/pF; PPAR\(_{\gamma}\) : 11.8±0.8 pA/pF; P=0.34; Figure 4C and 4D) or the current density of the inward rectifying current, I\(_{\text{K1}}\) (not shown), in the TG-PPAR\(_{\gamma}\) mice compared to WT mice. Cardiomyocytes had the same average size as shown by whole-cell membrane capacitance; the resting membrane potential and action potential amplitude (APA) were not significantly different in the PPAR\(_{\gamma}\) and WT littermate controls (online-only Data Supplement Table I).

The reduction in the I\(_{\text{K,slow}}\) current density in the TG-PPAR\(_{\gamma}\) mice was confirmed using a pharmacological approach. After exposure to 50 μmol/L 4-AP, WT cardiomyocytes showed a marked reduction in I\(_{\text{K,slow}}\) (Figure 5A). The 4-AP–sensitive I\(_{\text{K,slow}}\) current, however, was markedly reduced in cardiomyocytes from PPAR\(_{\gamma}\) mice (Figure 5A and 5B). Linear regression of the 4-AP–sensitive current demonstrated a significant difference in the slopes (pA · pF\(^{-1}\) · mV\(^{-1}\)) for the WT and PPAR\(_{\gamma}\) mice (WT: 0.211±0.002; PPAR\(_{\gamma}\) : 0.126±0.002; P<0.002; n=21 and 26, respectively). These data suggest that the prolongation of APD in PPAR\(_{\gamma}\) mice was primarily due to a reduction in I\(_{\text{K,slow}}\) current. We also measured the effect of 4-AP inhibition on APD concurrently by switching from voltage-clamp to current-clamp mode. Intracellular calcium was buffered to 10 mmol/L at a temperature of 22°C to optimize cardiomyocyte condition. Under these conditions, the APD in PPAR\(_{\gamma}\) was still prolonged. Blocking 4-AP–sensitive current in WT cardiomyocytes prolonged the APD to a larger extent than in PPAR\(_{\gamma}\) cardiomyocytes. After exposure to 4-AP, the APD was not significantly different in WT and PPAR\(_{\gamma}\) mice (Figure 5C).
Reduced Expression of Voltage-Dependent K⁺ Channels in PPARγ Hearts

Gene expression was determined by real-time quantitative polymerase chain reaction of RNA extracted from the ventricular tissue of 10- to 12-week-old mice. The messenger RNA (mRNA) expression of Kv,1.5, which contributes to I_K_slow, was significantly reduced in the TG-PPARγ mice compared with WT littermates. The mRNA expression of Kv,2.1, which encodes I_to,f was not significantly reduced. In contrast to larger animals, Kv,4.3 is not required for functional I_to,f in the mouse. Protein expression of the voltage-dependent K⁺ channels was measured using ventricular homogenates. In contrast to the modest or absent changes in mRNA expression, the protein expression of these channels was markedly reduced in the TG-PPARγ mice (Figure 6B and 6C). The protein levels of Kv,1.5 and Kv,2.1, which form I_K_slow in mouse, was significantly reduced to 67% and 50%, respectively, of WT littermate controls. These reductions in protein expression are consistent with the findings of reduced I_K_slow current density (Figure 5). The protein expression of Kv,4.2, which forms I_to,f, was also markedly reduced in the PPARγ mice, to 27% of the control level. Although the current density of I_to,f was reduced in the PPARγ mice, the reduction in protein expression of this subunit is far greater, suggesting upregulation of activity of the remaining Kv,4.2. The Kv,1.4 channel, which also contributes to I_to,f, is also reduced significantly.

Prolongation of APD Does Not Cause Triggered Activity

To better understand the mechanisms of ectopy and arrhythmia, ventricular myocytes were paced through the patch pipette in perforated configuration by current pulses (amplitude 0.1–0.4 nA, 3 ms duration) at intervals from 1000 to 250 ms. Under these conditions, we did not observe any early after-depolarizations (EADs) or delayed after-depolarizations (DADs) in PPARγ or WT cardiomyocytes (n=32, from 3 mice in each group).

Connexin43 Is Reduced but Conduction Velocity Is Normal

Connexin43 (Cx43, the major ventricular gap junction protein) mRNA and protein expression was significantly down-regulated in the PPARγ mice, to 31% (Figure 7A) and 14% (Figure 7B and 7C), respectively, of WT littermates. Other intercalated disk proteins, such as cadherin and plakoglobin, were also reduced (Figure 7C). Cx43 was decreased throughout the heart, as determined by immunohistochemistry of sections isolated from the anterior, lateral, and posterior walls of the LV and the right ventricle (Figure 7D). The reduction in Cx43 occurred before systolic dysfunction developed. To assess the effect of Cx43 reduction on ventricular impulse propagation, we optically mapped cardiac activation patterns in the PPARγ and WT littermate mice. Surprisingly, the minimum conduction velocity was not significantly reduced in the PPARγ mice compared with the control littermate mice (P=0.55 for LV; Figure 7F). Thus, it is not likely that the reduction in expression of Cx43 accounts for the increased propensity to develop ventricular arrhythmias and sudden death.

PPARγ Agonist Pioglitazone Increases Ventricular Arrhythmias in TG-PPARγ Mice

Transgenic PPARγ and WT littermates, 10 to 12 weeks of age, were fed standard mouse chow or pioglitazone-impregnated standard mouse chow for 3 weeks. Pioglitazone increased the mortality in the PPARγ mice (pioglitazone chow: 75% versus control chow: 25%, n=8 in each group) during the 3-week period but had no effect on mortality in the WT littermate animals (Figure 8A). To determine the mechanism(s) responsible for the pioglitazone-induced increased mortality in the TG-PPARγ mice, ECG telemeters were implanted into four 10-week-old TG-PPARγ mice and 4 WT littermate mice. Baseline telemetric measurements were initiated 4 days after implantation. Mice were then fed pioglitazone for 7 days. For TG-PPARγ mice, within 4 days of starting pioglitazone there was a significant increase in PVCs/h; by day 6 of pioglitazone chow, the number of PVCs/h was 15 times the baseline rate (P=0.0285 by U test). Complex ventricular ectopy also increased ~15-fold over the same time period (Figure 8B and 8C). Linear regression modeling of the time course shows a slope that is significantly different from zero (F test =0.017). Complex ventricular arrhythmias were never observed in the WT littermate.
mice in the absence or presence of pioglitazone ingestion, and even after 3 weeks of pioglitazone chow, the WT mice did not have an increase in PVCs/h. During the relatively short (and intermittent) monitoring period, sudden cardiac death was captured in 1 TG-PPAR\textsubscript{γ}/H9253 animal caused by spontaneous polymorphic ventricular tachycardia degenerating to ventricular fibrillation. We examined the ion channel protein expression from the hearts of TG-PPAR\textsubscript{γ}/H9253 mice fed either control or pioglitazone chow for 1 week. KV1.5, which encodes IK\textsubscript{slow}, was decreased by 35% (Figure 8C) in the pioglitazone-fed TG-PPAR\textsubscript{γ} mice compared with control chow–fed TG-PPAR\textsubscript{γ} mice (P=0.11 by U test). These results suggest that PPAR\textsubscript{γ} agonist–induced activation of PPAR\textsubscript{γ}, when overexpressed in the heart, has a deleterious effect on mortality and arrhythmogenesis.

**Discussion**

Despite the increasing prevalence of obesity and diabetes mellitus, little is known about the contribution of metabolic abnormalities to the pathophysiology of arrhythmias and

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**Figure 7.** Reduced Cx43 mRNA and protein expression in PPAR\textsubscript{γ} mice. A, Messenger RNA expression of Cx43, normalized to WT; n=4 mice in each group. *P<0.05 by U test. B, Immunoblot of Cx43 (with tubulin as a loading control) from mouse ventricular tissue; n=4 mice in each group. *P<0.05 by U test. C, Graph of Cx43 expression in PPAR\textsubscript{γ} mice, normalized to WT littermate control. D, Immunofluorescence of Cx43 protein (bright green) from indicated ventricular tissue. Scale bar: 75 μm. E, DAB staining for Cx43 (dark brown) from WT littermate and PPAR\textsubscript{γ} mice. Scale bar: 45 μm. F, Representative activation maps showing conduction across the ventricular epicardium of control and TG-PPAR\textsubscript{γ} mice. Scale bar: 1 mm. Lower, bar graph of LV and RV CV\textsubscript{min} for WT and PPAR\textsubscript{γ} mice. mRNA indicates messenger RNA; WT, wild type; PPAR\textsubscript{γ}, peroxisome proliferator–activated receptor γ; Ant, anterior; LV, left ventricle; Lat, lateral; Post, posterior; and RV, right ventricle.

**Figure 8.** Pioglitazone increases mortality and ventricular arrhythmias in TG-PPAR\textsubscript{γ} but not WT mice. A, Bar graph of 3-week mortality of TG-PPAR\textsubscript{γ} and WT mice fed either control chow or pioglitazone chow; n=8 in each group. B, Bar graph of average complex ventricular ectopy per hour in the PPAR\textsubscript{γ} mice fed control (white bars) and pioglitazone chow (black bars). Error bars are SEM; n=4 mice. C, Representative telemetry recordings of TG-PPAR\textsubscript{γ} mice fed pioglitazone showing nonsustained ventricular tachycardia. D, Bar graph of protein expression of Cx43 and KV channel subunits in TG-PPAR\textsubscript{γ} mice fed pioglitazone (black bars) relative to TG-PPAR\textsubscript{γ} mice fed control chow (white bars). Mean image intensity was normalized to tubulin signal and expressed as percentage of control chow fed TG-PPAR\textsubscript{γ}. Mean±SEM; n=4 animals in each group. PPAR\textsubscript{γ} indicates peroxisome proliferator–activated receptor γ; cont-chow, control chow; pio-chow, pioglitazone chow; and WT, wild type.
sudden cardiac death. Increased cardiomyocyte lipid stores are observed in obese and diabetic patients, and this may contribute to arrhythmias. In diabetic patients, plasma free fatty acid concentration correlates with the frequency of ventricular premature complexes, and patients with ischemic cardiomyopathy and obesity have more ventricular tachycardia than nonobese patients with ischemic cardiomyopathy.\textsuperscript{38,39} Diabetes mellitus and obesity are also associated with an increased risk of cardiomyopathy, independent of the presence of hypertension or coronary artery disease, which may represent a direct toxic effect of increased intracellular lipids.\textsuperscript{2,40} We used a gain-of-function approach to show that cardiomyocyte-specific metabolic derangements associated with the cardiac-specific overexpression of PPAR\textgreek{y} leads to ventricular arrhythmias and sudden cardiac death. The overexpression of PPAR\textgreek{y}, which can regulate transcription of numerous targets, and/or the subsequent cardiomyocyte lipid accumulation led to reductions in the expression and current density of key repolarizing currents, prolongation of the APD in vitro and in vivo, and the reduced expression of intercalated disc proteins. The electric remodeling of the heart ultimately caused a markedly increased incidence of malignant ventricular arrhythmias and sudden cardiac death, which occurred before the onset of systolic dysfunction but after the development of cardiomyocyte lipid accumulation. Treating the TG-PPAR\textgreek{y} mice but not WT littermate controls with a PPAR\textgreek{y} agonist increased the incidence of arrhythmias and mortality.

Although PPAR\textgreek{y} expression is normally relatively low in the heart, its expression is increased in several forms of heart disease including cardiomyopathy\textsuperscript{41} and cardiac hypertrophy\textsuperscript{42,43} and in the diabetic heart.\textsuperscript{44} Despite its low expression in the heart, tissue-specific loss of PPAR\textgreek{y} leads to cardiac hypertrophy with preserved systolic function.\textsuperscript{17} Peroxisome proliferation–activated receptor \textgreek{y} suppresses cardiac growth and embryonic gene expression and inhibits nuclear factor \textgreek{k}B activity in vivo\textsuperscript{47} and may also suppress inflammation.\textsuperscript{45} The cardiac metabolic abnormalities found in the TG-PPAR\textgreek{y} mice, specifically the lipid accumulation within cardiomyocytes,\textsuperscript{18} mimic many of the abnormalities found in the hearts of diabetic and obese/metabolic syndrome patients.\textsuperscript{19} The tissue-restricted TG overexpression of PPAR\textgreek{y} enables the preferential shunting of plasma triglycerides and fatty acids to the heart. Pioglitazone treatment in TG-PPAR\textgreek{y} mice would further enhance cardiac PPAR\textgreek{y} activity. The systemic effects of pioglitazone in WT mice, in contrast, by channeling a greater proportion of plasma triglycerides and fatty acids to adipose tissues, may actually reduce lipid uptake by the heart relative to the periphery.\textsuperscript{56} The differential activation of cardiac versus peripheral PPAR\textgreek{y} and the shunting and accumulation of lipid in either the heart or the periphery likely account for the TG phenotype and effects of pioglitazone. In WT animals, pioglitazone had no effect on mortality or incidence of ventricular tachyarrhythmias because pioglitazone activation of adipose-tissue PPAR\textgreek{y} predominates. In the TG-PPAR\textgreek{y} mice, although pioglitazone likely activated both cardiac and adipose PPAR\textgreek{y}, the level of expression of the cardiac PPAR\textgreek{y} likely favored additional cardiomyocyte lipid accumulation and increased electric remodeling, as reflected by an increased incidence of ventricular tachyarrhythmias, reduced expression of K\textgreek{+} channels, and increased mortality. In humans, the underlying ratio of adipose to tachyarrhythmias, reduced expression of K\textgreek{+} channels, and increased mortality. In humans, the underlying ratio of adipose to cardiac PPAR\textgreek{y} expression and the differential effects of different PPAR\textgreek{y} agonists (eg, pioglitazone versus rosiglitazone) likely modulates the beneficial versus toxic effects of these drugs on the heart.

The molecular mechanisms responsible for the reduction in K\textgreek{+} channel expression in the TG-PPAR\textgreek{y} mice are not known. The PPAR\textgreek{y} overexpression and lipid loading may exert their effects via transcriptional, translational, and/or post-translational processes. Cardiac-specific overexpression of PPAR\textgreek{\alpha}, a key regulator of diabetes mellitus–induced lipid metabolic dysregulation, also induced ion channel remodeling, predominantly a reduction of I\textsubscript{to,f} and a compensatory increase in I\textsubscript{K,slow}\textsuperscript{15}. No change in I\textsubscript{K,slow} current was found in young TG-PPAR\textgreek{\alpha} mice. In MHC-FATP TG mice, however, the I\textsubscript{peak} and I\textsubscript{K,slow} currents were selectively attenuated, without change in I\textsubscript{to,f} and I\textsubscript{K,slow}.\textsuperscript{14} In our study of the cardiac-specific overexpression of MHC-PPAR\textgreek{y} mice, I\textsubscript{peak} and I\textsubscript{K,slow} were significantly reduced, with a trend toward reduction in I\textsubscript{to,f} current. The remodeling of K\textgreek{+} channels in the TG-PPAR\textgreek{y} mice is similar to the remodeling found in the MHC-FATP mice. In the TG-PPAR\textgreek{\alpha} and TG-FATP mice, the electrophysiological phenotypes were less severe than the TG-PPAR\textgreek{y} mice, and sudden cardiac death (in nonpregnant animals) was not reported.\textsuperscript{13,14} Different transcriptional and/or posttranscriptional pathways may be modified in these mice, which causes changes in distinct K\textgreek{+} currents. In all 3 animal models, fatty-acid accumulation and utilization are increased. Glucose uptake and metabolism are decreased in the MHC-FATP and MHC-PPAR\textgreek{\alpha} mice whereas in the MHC-PPAR\textgreek{y} mice glucose uptake is increased.

Cardiac-specific loss of Cx43 is associated with significant conduction slowing and a higher propensity for the development of ventricular arrhythmias and sudden death.\textsuperscript{24,26} Large reductions of Cx43 expression levels are required to significantly affect epicardial conduction velocities. A 50% reduction of Cx43 protein does not produce significant conduction slowing, but a 70% to 95% reduction of Cx43 protein results in reduced conduction velocity, increased dispersion of conduction, and enhanced arrhythmogenicity.\textsuperscript{47–49} The ≈12-week-old TG-PPAR\textgreek{y} mice demonstrate ≈70% reduction in Cx43 mRNA and ≈85% reduction in Cx43 protein amounts. The lack of significant slowing of the conduction velocity in the TG-PPAR\textgreek{y} mice suggests that other proteins or posttranslational modifications must be compensating for the marked reduction in Cx43 protein levels. Furthermore, the finding of normal conduction velocity implies that the reduction in Cx43 protein in the TG-PPAR\textgreek{y} mice does not contribute significantly to the increased incidence of ventricular arrhythmias and mortality. The wide QRS may be due to damage to the His-Purkinje system rather than reduced Cx43 levels. The reduced levels of other intercalated disk proteins is probably not a direct effect of PPAR\textgreek{y} because in some cancer models, PPAR\textgreek{y} overexpression increases cadherins.\textsuperscript{50} The reduction in intercalated disk proteins may contribute to the reduction in Cx43 protein.
In summary, we have found that overexpression of PPARγ in the heart is sufficient to induce action potential remodeling and to cause an acquired long-QT syndrome phenotype. The reduced repolarization reserve increases the incidence of spontaneous ventricular arrhythmias and sudden death. Pioglitazone treatment increases the incidence of complex ventricular arrhythmias and sudden death in the TG-PPARγ mouse but not in WT littermates. Although an important limitation of this work is that the ion channels responsible for cardiac-action-potential repolarization in mice are different than in humans, the TG-PPARγ mouse recapitulates an arrhythmic phenotype observed in patients with diabetes mellitus and metabolic syndrome.

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Disclosures
None.

References
Diabetes mellitus and obesity confer an increased risk of sudden cardiac death and are associated with cardiomyocyte lipid accumulation and altered cardiac electric properties (demonstrated by prolongation of the QRS and QT intervals). In order to study the effects of metabolic abnormalities on arrhythmias without the complex systemic effects of diabetes mellitus and obesity, we studied a mouse model with cardiac-specific overexpression of peroxisome proliferator–activated receptor γ (PPARγ), a transcription factor that is a key regulator of glucose and lipid metabolism. These PPARγ transgenic mice develop abnormal accumulation of intracellular lipids and die as young adults, before any significant reduction in systolic function. We found that these mice have prolongation of the QT interval and spontaneous ventricular arrhythmias, including polymorphic ventricular tachycardia and ventricular fibrillation. Isolated cardiomyocytes demonstrated prolonged action potential duration caused by reduced potassium currents, which are responsible for repolarization. Short-term exposure to pioglitazone, a PPARγ agonist, had no effect on mortality or rhythm in wild-type mice but further exacerbated the arrhythmic phenotype and increased mortality in the PPARγ mice. Our findings support an important link between PPARγ activation, cardiomyocyte lipid accumulation, ion channel remodeling, and increased cardiac mortality. This mouse model may help identify the molecular mechanisms leading to sudden death in diabetic and/or obese patients.

Clinical Perspective

Morrow et al  PPARγ Overexpression Induces Fatal Arrhythmias  2821
Mice With Cardiac Overexpression of Peroxisome Proliferator–Activated Receptor γ Have Impaired Repolarization and Spontaneous Fatal Ventricular Arrhythmias

John P. Morrow, Alexander Katchman, Ni-Huiping Son, Chad M. Trent, Raffay Khan, Takayuki Shiomi, Haiyan Huang, Vaibhav Amin, Joshua M. Lader, Carolina Vasquez, Gregory E. Morley, Jeanine D'Armiento, Shunichi Homma, Ira J. Goldberg and Steven O. Marx

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Mice with cardiac overexpression of PPARγ have impaired repolarization and spontaneous fatal ventricular arrhythmias

SUPPLEMENTAL MATERIAL

Supplemental Online Methods

Animal Care and Breeding
The PPARγ transgenic mouse has cardiac-specific overexpression of PPARγ driven by the myosin heavy chain (MHC) promoter as previously described 1. Animal protocols were approved by the Columbia University Institutional Animal Care and Use Committee and were carried out in accordance with the NIH guidelines for the care and use of laboratory animals. Pioglitazone-chow (30 mg/kg) was purchased from Research Diets2.

Telemetry and ECG analysis
Telemetry devices (Data Sciences International, model EA-F20) were implanted in 10 week-old mice using inhaled isoflurane anesthesia. The two subcutaneous leads were positioned to approximate limb lead II of a human ECG. The mice recovered for one week after surgery before initiating 24-hour recordings. ECG intervals were measured manually, blinded to genotype, using Ponemah 3 software from recordings with minimal artifact at heart rates of 520-550 bpm to avoid the issues of correcting QT for rate. Intervals were averaged from 4 consecutive beats. PVC and arrhythmias counts were tallied manually by review of daily telemetry recordings. Daily heart rates were averaged for 3 animals in each group.

Echocardiography
Transthoracic echocardiography was performed using a high-resolution imaging system with a 30-MHz imaging transducer (Vevo 770; VisualSonics, Toronto, ON, Canada). The mice were anesthetized with isoflurane throughout the procedure. Care was taken to minimize sedation by monitoring the heart rate and respiratory rate of the mice. Images were obtained using short-axis views at the level of papillary muscles, and each parameter was measured using M-mode view. Images were recorded in a digital format and were then analyzed off-line. Percent fractional shortening (%FS) was calculated as follows: %FS = (LVDd-LVDs)/LVDd X 100, where LVDd is left ventricular diastolic dimension and LVDs is left ventricular systolic dimension.

Isolation of Cardiomyocytes and Cellular Electrophysiology
Cardiomyocytes were isolated using methods previously described 3. Briefly, the mouse heart was removed and the aorta was cannulated. After perfusing Ca^{2+}-free buffer for two min, a mixture of collagenase and protease was then perfused through the coronary arteries for 5-7 min (Blendzyme 4 or Liberase TH, 0.3 mg/mL, Roche) at a [Ca^{2+}] =12.5 µM. The LV tissue was teased apart with fine forceps and briefly pipetted to release individual cells. After enzymatic dispersion, [Ca^{2+}] in the buffer containing 3.5 mg/ml BSA was elevated in 4 steps up to 0.8 mM. Cells were transferred into experimental temperature controlled chamber (Delta T Culture Dish, Bioptechs Inc). Only non-contracting rod shaped cells with clear striations were used in this study. Experiments were performed on freshly isolated cardiomyocytes from left ventricle of PPARγ mice (10 animals) and their littermate controls (12 animals).
Membrane currents were measured by the whole-cell patch-clamp method using a MultiClamp 700B amplifier (Axon Instruments, Union City, CA). For action potential duration (APD) measurements (Fig. 3), perforated, whole-cell patch-clamp was utilized, using amphotericin B (300 µg/ml; Sigma A9528) at 35°C. Micropipettes were pulled from borosilicate glass capillaries (BF150-110-7.5, Sutter Instruments, Novato CA) on a programmable horizontal puller (S-97; Sutter Instruments, Novato CA). The pipettes had inner tip diameters of about 1 to 1.5 µm. When filled with internal solutions, they had resistances of 1.5 to 2.5 MΩ. Data were filtered at 4 KHz with a four-pole low-pass Bessel filter and sampled at 10 KHz. All experiments were performed using pCLAMP 10.2 software (Axon Instruments, Union City CA). Boltzmann’s fits were performed as previously described.

To record action potentials and K⁺ currents, the pipette solution contained 130 mM K⁺ gluconate, 10 mM NaCl, 10 mM EGTA, 1 mM MgCl₂, 2 mM Mg-ATP, 2.0 mM CaCl₂ and 10 mM HEPES, adjusted to pH 7.2 with KOH. Cells were superfused at room temperature with HEPES-buffered Tyrode’s containing 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with NaOH). Voltage clamp correction for a liquid junction potential of -13.6 mV was made by configuring the recording files in CLAMPEX of pCLAMP 10.2. Series resistances were usually less than 2 MΩ after 60% compensation. All voltages in current clamp recording were also corrected for the junction potential.

The decay phases of the outward K⁺ currents evoked during 4.0 s depolarizing voltage steps were fitted by a double exponential function of the form:

\[ Y(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + B, \]

where \( t \) is time, \( \tau_1 \) and \( \tau_2 \) are the decay time constants, \( A_1 \) and \( A_2 \) are the amplitudes of the inactivating current components (\( I_{\text{to},1} \) and \( I_{\text{K,slow}} \)), and \( B \) is the amplitude of the non-inactivating current component, \( I_{\text{ss}} \).

To study L-type Ca²⁺ current, we used a bath solution containing 140 mM TEA-Cl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with CsOH). In these experiments, pipettes were filled with solution contained 135 mM CsCl, 10 mM EGTA, 1 mM MgCl₂, 2 mM Mg-ATP, 2.0 mM CaCl₂ and 10 mM HEPES, adjusted to pH 7.2 with CsOH. Series resistance was usually less than 2 MΩ after 60% compensation. Leak currents and capacitance transients were subtracted by a P/4 protocol. Voltage clamp correction for a liquid junction potential of -3.6 mV was made by configuring the recording files in CLAMPEX of pCLAMP 10.2. To evaluate the steady state activation of Ca²⁺ currents, the cell membrane potential was held at -70 mV and stepped for 450 ms to -60 to +50 mV in 10 mV increments. The interval between pulses was 10 s. To study the steady state inactivation of Ca²⁺ currents, the cell membrane potential was held at -70 mV and stepped to -70 to +30 mV for 650 ms in 10 mV increments and then stepped to the test potential +10 mV for 650 ms. The interval between pulses was 10 sec. Na⁺ currents were measured as described previously.

Action potential parameters, K⁺, Na⁺ and Ca²⁺ currents were measured and analyzed using pCLAMP 10.2, Excel and Origin 7.5 (Originlab, Northampton, MA) software. \( V_{1/2} \) and \( k \) were calculated from Boltzmann function fitting for each cell. Statistical significance of observed differences were evaluated using TTEST (p<0.05). Regression analysis was done using GraphPad Software.

Real-time PCR
Samples of ventricular tissue from PPARγ mice and WT littermates, at 10-12 weeks of age, were used for harvesting RNA for RT-PCR. Cardiac tissue was homogenized with a Mini-BeadBeeater...
RNA was then purified using a Qiagen RNeasy kit (item 74104). cDNA was synthesized using the Applied Biosystems high capacity RNA to cDNA kit (#4387406) and diluted to 10 ng/μL for use as a template (20 ng template was used for each 20 μL reaction). Real-time PCR was performed using an Applied Biosystems StepOne Plus Real-Time PCR system with StepOne Software v2.0 and inventoried primers from Applied Biosystems. PCR was performed for 40 cycles with automated detection of crossing threshold. PCR reactions were performed with duplicate wells with actin as a control reaction and no-template lanes for negative controls.

**Immunoblots**
Cardiac lysates were made by homogenizing ventricular tissue in buffer containing 1% (v/v) Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, phosphatase inhibitor cocktail, and protease inhibitors (complete mini-tablet, calpain I and II inhibitors, Roche). Lysates were used for PAGE and then transferred to nitrocellulose membranes for immunoblots. The following antibodies were used: anti-Cx43 (Invitrogen), anti-Kv1.5 and anti-Kv2.1 (Alomone), anti-Kv4.2 and anti-Kv1.4 (Thermo-Scientific Pierce), and anti-tubulin (Santa Cruz Biotechnology). Chemiluminescence signal was obtained using a Kodak Image Station 400R Pro digital camera with Kodak Molecular Imaging Software v4.5.1. Signal intensity was quantified using ImageJ software (NIH). Blots for tubulin were performed to normalize loading of lanes, using the same membrane.

**Immunohistochemistry**
Heart tissue was fixed with 4% paraformaldehyde, embedded in paraffin wax, and then sectioned. Sections were deparaffinized and underwent antigen retrieval treatment (autoclaved with pH 9.0 Tris-buffer at 121˚C for 15 min). For DAB staining, sections were treated with 0.3% H2O2 to block endogenous peroxidase. Sections were incubated with rabbit polyclonal antibodies against Cx43 (1:200 dilution, Invitrogen Corp.) or non-immune rabbit polyclonal IgG at the same concentration, at 4˚C overnight. For DAB staining, after exposure to anti-rabbit swine antibody conjugated to biotin (1:500, DakoCytomation Denmark A/S, Glostrup, Denmark) for 1 hr in room temperature, the sections were treated with peroxidase-labeled ABC (VECTASATIN ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and developed with DAB solution (ImmPACT DAB Peroxidase Substrate, Vector Laboratories, Inc.). After the reaction, the sections were counterstained with hematoxylin and observed by a light microscope. For immunofluorescent staining, after reaction with anti-rabbit donkey antibody conjugated to Alexa Fluor 488 (1:500, Invitrogen Corp.), the sections were counterstained with DAPI and observed by a fluorescent microscope. Tissues from two mice in each group (PPARγ overexpression and WT littermates) were used. Slides were photographed with a digital camera. Signal intensity was quantified using ImageJ software (NIH).
Supplement References:


Supplemental Figure 1. Heart weight, body weight and heart weight:body weight ratio for WT and PPARγ mice. Mean ± SEM. n=8, * indicates p<0.05 by U-test.
<table>
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<tr>
<th></th>
<th>WT mean, pF</th>
<th>SEM</th>
<th>WT mean, pF</th>
<th>SEM</th>
<th>PPARγ mean, pF</th>
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<td>Whole-cell membrane capacitance</td>
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<td>1.5</td>
<td>112.3</td>
<td>1.3</td>
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**Supplemental Table 1.** Additional patch-clamp data. WT: n=43; PPARγ: n=31. None of the comparisons were statistically significant by t-test.