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Cellular remodeling in heart failure disrupts K_{ATP} channel-dependent stress tolerance

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ATP-sensitive potassium (K_{ATP}) channels are required for maintenance of homeostasis during the metabolically demanding adaptive response to stress. However, in disease, the effect of cellular remodeling on K_{ATP} channel behavior and associated tolerance to metabolic insult is unknown. Here, transgenic expression of tumor necrosis factor α induced heart failure with typical cardiac structural and energetic alterations. In this paradigm of disease remodeling, K_{ATP} channels responded aberrantly to metabolic signals despite intact intrinsic channel properties, implicating defects proximal to the channel. Indeed, cardiomyocytes from failing hearts exhibited mitochondrial and creatine kinase deficits, and thus a reduced potential for metabolic signal generation and transmission. Consequently, K_{ATP} channels failed to properly translate cellular distress under metabolic challenge into a protective membrane response. Failing hearts were excessively vulnerable to metabolic insult, demonstrating cardiomyocyte calcium loading and myofibrillar contraction banding, with tolerance improved by K_{ATP} channel openers. Thus, disease-induced K_{ATP} channel metabolic dysregulation is a contributor to the pathobiology of heart failure, illustrating a mechanism for acquired channelopathy.

Keywords: ATP-sensitive potassium channel/energy metabolism/heart failure/potassium channel openers/TNFα

Introduction

Stress occurs as a threat to the physiological parameters necessary for survival, with the adaptive response incorporating alterations in bodily functions to sustain the intensified performance level necessary for confrontation or evasion. A critical component in maintaining homeostasis during this metabolically demanding adaptive reaction is the ATP-sensitive potassium (K_{ATP}) channel (Zingman *et al*., 2002a), a high-fidelity metabolic sensor that adjusts membrane potential-dependent cell functions to match metabolic state (Weiss and Lamp, 1987; Ashcroft, 1988; O'Rourke *et al*., 1994; Nichols *et al*., 1996). K_{ATP} channels are broadly represented in tissues that propagate the general adaptation reaction to stress, including nervous system (Amoroso *et al*., 1990; Miki *et al*., 2001; Yamada *et al*., 2001), vasculature (Yamada *et al*., 1997; Chutkow *et al*., 2002; Miki *et al*., 2002), heart (Inagaki *et al*., 1996), skeletal muscle (Vivaudou *et al*., 1991; Allard and Lazdunski, 1992) and pancreatic β-cells (Ashcroft *et al*., 1984; Inagaki *et al*., 1995; Koster *et al*., 2000; Aguilar-Bryan *et al*., 2001). While tissue-specific functions of K_{ATP} channels arise through distinctive properties of subunit isoforms, the channel role as membrane metabolic mediator is ubiquitous (Seino and Miki, 2003; Zingman *et al*., 2003).

In the heart, where K_{ATP} channels were originally discovered (Noma, 1983), the multimeric channel complex is assembled by physical association of the pore-forming Kir6.2 and regulatory SUR2A subunits (Inagaki *et al*., 1996; Lorenz and Terzic, 1999). Metabolic sensing occurs through modulation of the ATP sensitivity of Kir6.2 (Tucker *et al*., 1997) by the SUR2A subunit that harbors an intrinsic ATPase activity such that stabilization of SUR2A in a post-hydrolytic state favors K^+ efflux through Kir6.2, leading to membrane hyperpolarization (Bienengraeber *et al*., 2000; Zingman *et al*., 2001, 2002b; Matsuo *et al*., 2002; Matsushita *et al*., 2002). Integration of nucleotide-dependent K_{ATP} channel gating with cellular metabolism occurs through interaction with membrane and cytosolic modulators (Shyng and Nichols, 1998; Beguin *et al*., 1999; Lin *et al*., 2000; Carrasco *et al*., 2001; Abraham *et al*., 2002; Crawford *et al*., 2002a). In particular, creatine kinase-catalyzed phosphotransfer bridges diffusional barriers between mitochondrial ATP production and cellular ATP-sensitive processes, securing over 90% of energetic distribution in the heart and supporting myocardial tolerance to metabolic insult (Wallimann *et al*., 1992; Dzeja *et al*., 1999; Saupe *et al*., 2000; Pucar *et al*., 2001; Crozatier *et al*., 2002). Indeed, the intimate relationship between the ATPase activity of SUR2A and creatine kinase phosphotransfer is fundamental to cardiac K_{ATP} channel translation of metabolic fluctuations into homeostatic changes in membrane excitability (Dzeja and Terzic, 1998; Bienengraeber *et al*., 2000; Zingman *et al*., 2001; Abraham *et al*., 2002; Crawford *et al*., 2002b).

The role of K_{ATP} channels as mediators of the response to stress is underscored in mice with genetic deletion of Kir6.2 that underperform on exercise stress test, a natural trigger of the general adaptation syndrome (Zingman *et al*., 2002a, 2003) The contribution of cardiac K_{ATP} channels is demonstrated by failure of Kir6.2 knockout hearts to

shorten action potentials or to sustain augmented contractile performance, suffering Ca^{2+} overload, injury and increased susceptibility to fatal arrhythmia under adrenergic stress (Zingman *et al*., 2002a), with reduced tolerance to ischemic challenge (Suzuki *et al*., 2002). Furthermore, congenital K_{ATP} channel mutations lead to abnormal metabolic behavior, as has been shown in pancreatic β-cells (Thomas *et al*., 1995; Nichols *et al*., 1996; Aguilar-Bryan *et al*., 2001), and in heart, while no spontaneous mutations have been reported, engineered K_{ATP} channel mutants with abnormal sensitivity to ATP (Rajashree *et al*., 2002) or altered metabolic signaling to K_{ATP} channels by genetic deletion of creatine kinase (Abraham *et al*., 2002) respond aberrantly to myocardial metabolic inhibition. Yet, while the K_{ATP} channel metabolic integration and associated homeostatic role are increasingly elucidated, it is unestablished whether disease states due to an altered cellular environment will modify K_{ATP} channel behavior and the ability of diseased organisms to adapt to stress.

Cellular remodeling with consequent dysfunction of cellular processes is a fundamental feature of disease conditions. A paradigm of this principle is cardiomyocyte modification in heart failure. Structural and energetic remodeling of cardiomyocytes in failing hearts occurs in response to the altered mechanical, neurohumoral and/or inflammatory environment, and eventually becomes maladaptive, precipitating electrical and mechanical dysfunction (Towbin and Bowles, 2002). In this regard, remodeling in heart failure manifests with deficits in creatine kinase phosphotransfer (Liao *et al*., 1996; Nascimben *et al*., 1996; Tian *et al*., 1996; Neubauer *et al*., 1997; DeSousa *et al*., 1999; Dzeja *et al*., 2000; Ye *et al*., 2001), but it is unknown whether K_{ATP} channel gating and associated myocyte tolerance to stress is affected. This is of particular significance as cardiomyocytes in heart failure confront the metabolic insults of hypoxia, ischemia and adrenergic toxicity (Braunwald and Bristow, 2000; Bradham *et al*., 2002; Towbin and Bowles, 2002), such that altered stress tolerance would impact disease progression.

Therefore, to assess K_{ATP} channel behavior in a disease state, a model of heart failure, induced by the cytokine tumor necrosis factor-α (TNFα), was utilized. In normal heart, TNFα is not expressed; however, in heart failure, circulating TNFα levels correlate with disease severity and mortality (Levine *et al*., 1990; Torre-Amione *et al*., 1996; Deswal *et al*., 2001). Cardiac TNFα expression is triggered by biomechanical stress, and through cross-linkage of cardiomyocyte membrane receptors activates multiple kinases and transcriptional regulators that modulate growth and differentiation (McTiernan and Feldman, 2000; Mann, 2003). The role of this cytokine in promotion of cardiac remodeling is further evidenced by induction of dilated cardiomyopathy in response to constitutive cardiac TNFα overexpression (Bryant *et al*., 1998; Kadokami *et al*., 2000; Sivasubramanian *et al*., 2001). Here, we find in heart failure, induced by transgenic expression of TNFα, that cardiomyocyte remodeling did not affect intrinsic K_{ATP} channel properties, but hindered its metabolic regulation through alteration of signal communication. As a result K_{ATP} channels did not recognize cellular stress, resulting in failure of their homeostatic function. While the underlying signaling defects in heart failure created vulnerability to stress, tolerance was improved by direct pharmacological targeting of K_{ATP} channel proteins.

Results

Transgenic cardiac TNFα expression recapitulates heart failure

Mice engineered to overexpress TNFα (TNFα-TG) in the heart (Figure 1A; Sivasubramanian *et al*., 2001) were unable to match the physical exertion of wild-type (WT) littermates (Figure 1B). By 8–12 weeks of age, on treadmill, TNFα-TG dropped out earlier and at lower workloads than simultaneously exercised WT ($n = 6$ each, $p < 0.05$; Figure 1B). Tolerated workload was 34.3 ± 5.5 and 56.1 ± 2.7 J in TNFα-TG ($n = 6$) and WT ($n = 6$), respectively ($p < 0.05$, Figure 1B, inset). On echocardiography, left ventricular fractional shortening was significantly less in TNFα-TG (35.5 ± 2.1%, $n = 3$) than in WT (45.3 ± 2.4%, $n = 4$; $p < 0.05$; Figure 1C), despite similar heart rates (470 ± 16 b.p.m., $n = 3$ and 456 ± 37 b.p.m., $n = 4$, respectively). Under β-adrenergic challenge with isoproterenol, TNFα-TG ($n = 3$) failed to augment left ventricular fractional shortening (39 ± 3%) compared with the response in WT (85 ± 8%, $n = 4$; $p < 0.05$; Figure 1C), although increases in heart rate were similar (520 ± 29 and 518 ± 20 b.p.m., respectively). While there was no spontaneous death in WT mice observed for up to 53 weeks, TNFα-TG median survival was 26 weeks (Figure 1D). Remodeling of TNFα-TG myocardium resulted in dilated cardiomyopathy with chamber dilatation and left ventricular wall thinning (Figure 1E, top), cardiomyocyte ultrastructural deformation and irregular surface morphology (Figure 1E, middle), and myofibrillar disorganization (Figure 1E, bottom). Thus, exercise intolerance, ventricular dysfunction, compromised survival, with chamber and cardiomyocyte remodeling, key features of heart failure, were recapitulated in the transgenic TNFα heart failure model.

K_{ATP} channels retain intrinsic gating properties but receive defective metabolic signaling in failing hearts

To distinguish intrinsic channel properties from the regulatory contribution of the cellular milieu, K_{ATP} channel behavior was compared in excised membrane patches versus the open cell-attached configuration that allows retention of cellular infrastructure, including organelles and metabolic enzymes, while permitting exchange of ions and metabolic ligands. In membrane patches excised from the cell environment, the kinetic behavior within a burst of K_{ATP} channel opening (Figure 2A), the channel current–voltage relationship (Figure 2B) and the concentration dependence to ATP (IC_{50} = 31 ± 3 versus 37 ± 3 μM; Figure 2C) were virtually identical for TNFα-TG ($n = 4$) and WT ($n = 3$). While cytosolic ATP levels were preserved in the TNFα-TG compared with the WT (5.4 ± 0.2 versus 5.8 ± 0.3 mM; $n = 4$ each), and no spontaneous channel activity was observed in unpermeabilized TNFα-TG or WT cardiomyocytes, in open cell-attached patches the concentration dependence of K_{ATP} channel activity to ATP was significantly altered in TNFα-TG compared with WT (Figure 2D and E). Applied ATP (0.1–0.5 mM)

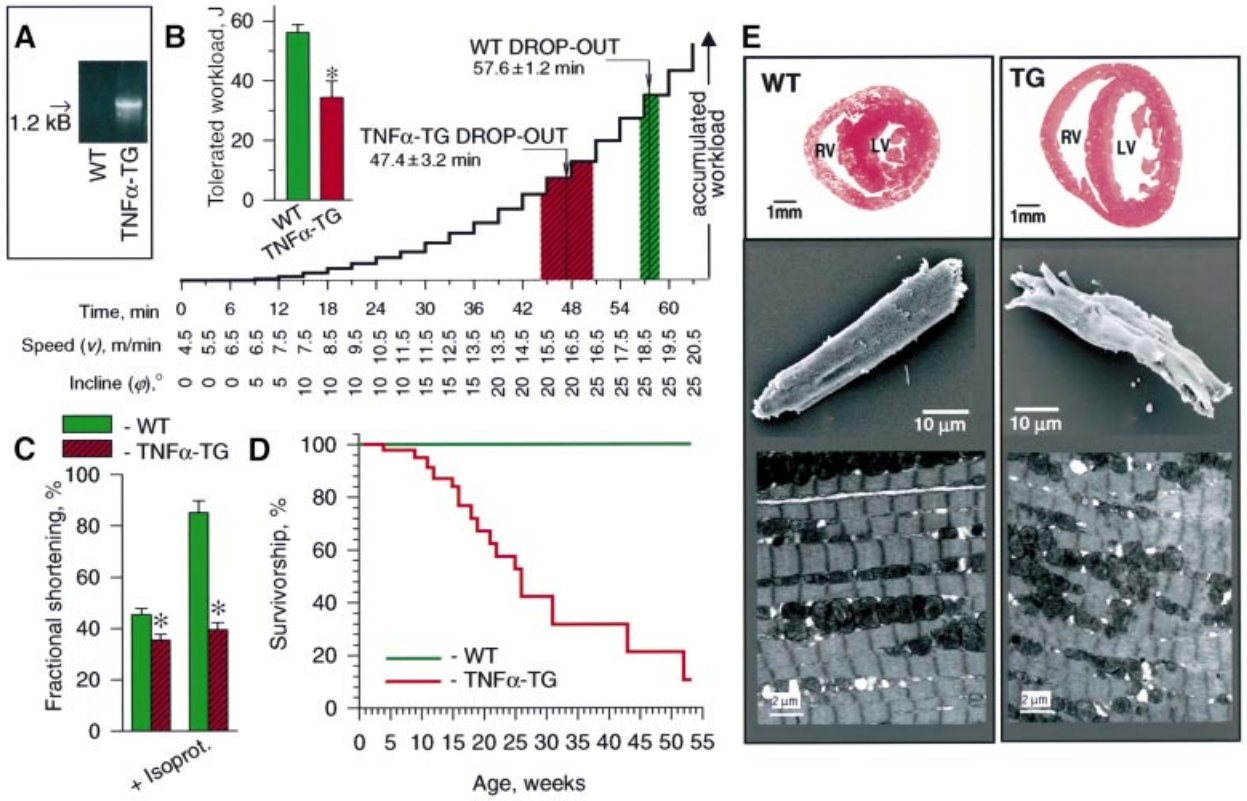

Fig. 1. Heart failure in TNFα transgenic mice. (**A**) The 1.2 kB band TNFα transgene in tail-cut PCR of transgenic (TNFα-TG) but not WT mice. (**B**) Exercise intolerance of TNFα-TG, compared with WT, with lower tolerated workload (inset) and earlier treadmill drop-out ($p < 0.05$). (**C**) Left ventricular fractional shortening, by echocardiography, was significantly less in TNFα-TG than WT ($p < 0.05$). In mice challenged with isoproterenol (0.5 μg i.p.), augmentation of fractional shortening was greater in WT compared with TNFα-TG ($p < 0.05$). (**D**) Mortality was greater in TNFα-TG (initial $n = 135$, 85% censored by 53 weeks) compared with WT (initial $n = 175$, 99% censored by 53 weeks) mice ($p < 0.05$). (**E**) Remodeling in 8-week-old TNFα-TG (TG) mice. Top: chamber dilation and reduced wall thickness at the base of TG versus WT hearts. Middle: distortion of architecture in TG versus rod-shaped WT ventricular cardiomyocytes on scanning microscopy. Bottom: myofibrillar disorganization in TG versus WT ventricular tissue by transmission electron microscopy.

effectively blocked channel activity in WT, but not in TNFα-TG (Figure 2E), and consequently the concentration–response curves defining the ATP sensitivity of K_{ATP} channels were significantly different between WT ($n = 3$) and TNFα-TG ($n = 3$), with an IC_{50} of 66 ± 5 and 443 ± 30 μM, respectively ($p < 0.05$; Figure 2D). Thus, although the biophysical properties of K_{ATP} channels were intact and excised channels properly measured ATP, within the cellular milieu of the failing cardiomyocyte recognition by the channel of this major metabolic ligand was altered.

Energetic signaling to K_{ATP} channels principally depends on generation of ATP by oxidative phosphorylation in concert with high-energy phosphoryl transfer through the creatine kinase system (Sasaki *et al.*, 2001; Abraham *et al.*, 2002; Crawford *et al.*, 2002b). Here, mitochondria from TNFα-TG hearts had depressed ADP-stimulated respiration, a measure of oxidative phosphorylation potential (Ozcan *et al.*, 2002), with 197 ± 18 ng atoms O/mg/min ($n = 7$) utilized versus 394 ± 22 ng atoms O/mg/min ($n = 7$) in WT ($p < 0.05$; Figure 3A). Glycogen, a substrate reservoir for oxidative phosphorylation (Goodwin *et al.*, 1996), was abundant in electron micrographs of WT hearts (68 ± 18 per μm^2, $n = 3$) forming profuse electron-dense particles (Figure 3B, left). However, glycogen granules were less common in TNFα-TG (12 ± 9 per μm^2, $n = 3$) myocardium ($p < 0.05$; Figure 3B, right). Moreover, creatine kinase flux in TNFα-TG ($n = 3$) was significantly lower than in WT ($n = 4$) hearts (192 ± 14 versus 291 ± 22 nmol/mg/min, $p < 0.05$; Figure 3C), with the responsiveness of K_{ATP} channels to creatine kinase signaling, induced in the open cell-attached mode by application of its substrate creatine phosphate, blunted in TNFα-TG cardiomyocytes (Figure 3D). Indeed, the channel IC_{50} to creatine phosphate was 250 ± 18 μM ($n = 3$) in TNFα-TG versus 94 ± 5 μM ($n = 3$) in the WT ($p < 0.05$; Figure 3E). Thus, cardiomyocytes from failing myocardium displayed a deficit in the potential for metabolic signal production and transmission responsible for optimal K_{ATP} channel regulation.

Dysregulated K_{ATP} channels unable to adjust membrane excitability under stress in cardiomyocytes of failing hearts

The deficit in the potential for energetic communication in the remodeled TNFα-TG cardiomyocyte could indicate impaired integration of K_{ATP} channels with the cellular environment. This would hamper the delivery of metabolic stress-induced signals. Yet, proper K_{ATP} channel response

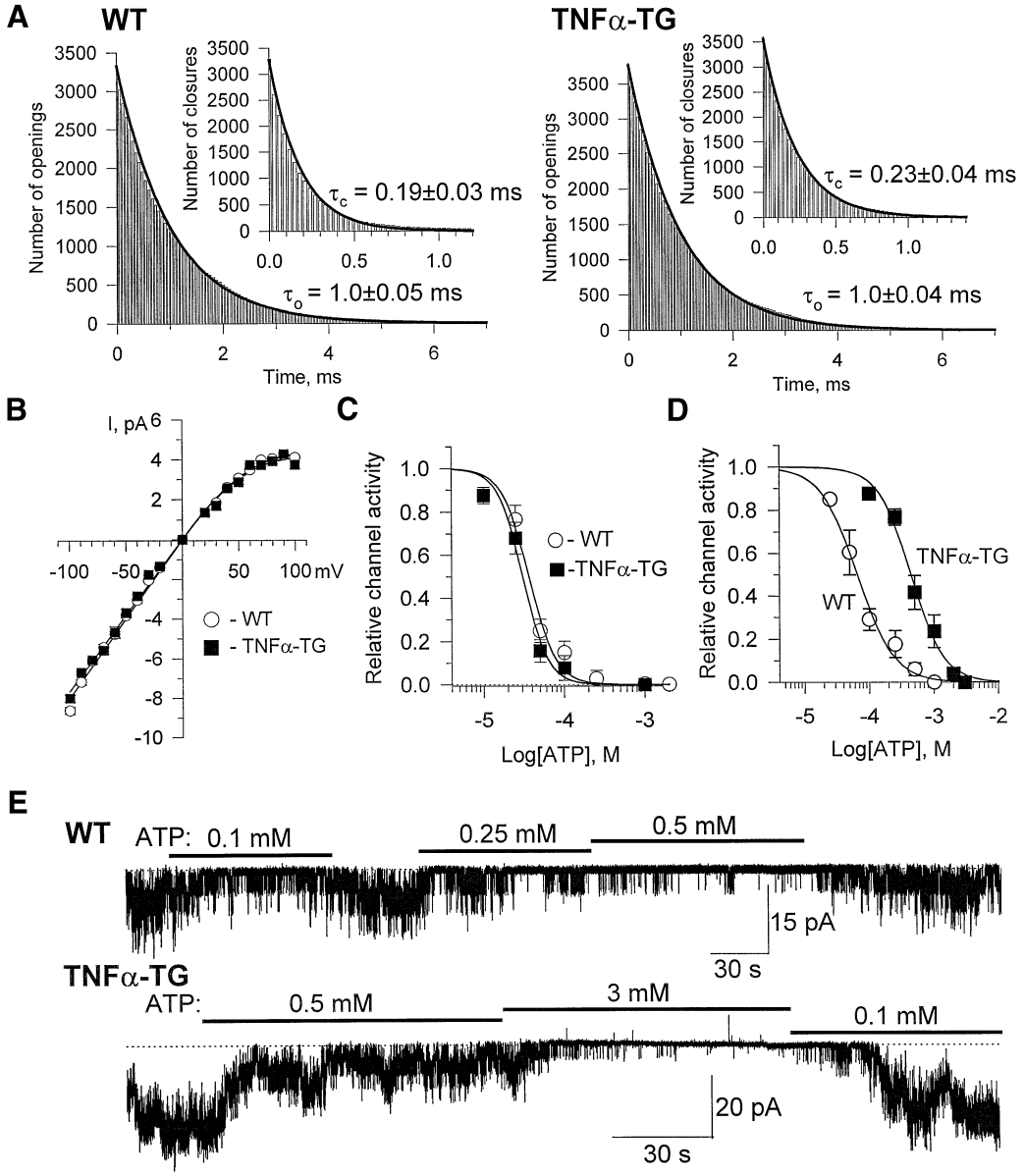

Fig. 2. Intact K_{ATP} channels in TNFα-TG cardiomyocytes receive altered ATP signal. (**A**) Intraburst K_{ATP} channel kinetics were indistinguishable in excised WT and TNFα-TG patches, with characteristic open and closed times (τ_o and τ_c), derived from the best-fit of corresponding distributions, not significantly different ($n = 3$). (**B**) K_{ATP} channel current–voltage relationships ($n = 4$) with identical channel conductance and rectification in WT and TNFα-TG patches. (**C**) K_{ATP} channel activity in excised membrane patches calculated relative to activity in the absence of ATP, and fitted by the Hill equation $1/[1 = x/IC_{50}]^k$ (solid curves), where x is ATP concentration, h the Hill coefficient and IC_{50} the half-maximal inhibition concentration. (**D** and **E**) K_{ATP} channels, in open cell-attached mode, show altered effect of ATP in TNFα-TG compared with WT.

is required for cellular homeostasis under stress, with channel opening responsible for protective shortening of the cardiac action potential (Nichols and Lederer, 1991; Rajashree *et al*., 2002; Suzuki *et al*., 2002; Zingman *et al*., 2002a). Here, hypoxic stress, simulated by the mitochondrial uncoupler dinitrophenol (DNP), induced vigorous K_{ATP} channel opening in cardiomyocytes from WT, but not TNFα-TG hearts (Figure 4A). On average, K_{ATP} channel activity was 4-fold higher in WT ($n = 3$) than TNFα-TG ($n = 3$; Figure 4B), with DNP-induced channel opening abolished by the mitochondrial F_0F_1-ATPase inhibitor oligomycin (1 μg/ml). Thus, K_{ATP} channels in the failing cardiomyocyte did not appropriately recognize metabolic stress. As a consequence, monophasic action potential shortening in hypoxia was blunted in TNFα-TG compared with WT hearts (Figure 4C). By 3 min into hypoxia, monophasic action potential duration at 90% repolarization (APD_{90}) was significantly shorter ($p < 0.05$) in WT (27 ± 1 ms, $n = 3$) than in TNFα-TG hearts (47 ± 2 ms; $n = 4$), despite similar pre-hypoxic APD_{90} (52 ± 6 versus 57 ± 2 ms, $p > 0.05$; Figure 4D). Therefore, in the failing heart, K_{ATP} channels behaved as if uncoupled from cellular metabolic signals, compromising protective K_{ATP} channel-dependent regulation of membrane excitability under stress.

K_{ATP} channel openers restore tolerance to stress

Hearts lacking K_{ATP} channels are susceptible to adrenergic stress-induced calcium overload and associated myocyte injury (Zingman *et al*., 2002a). Here, WT cardiomyocytes

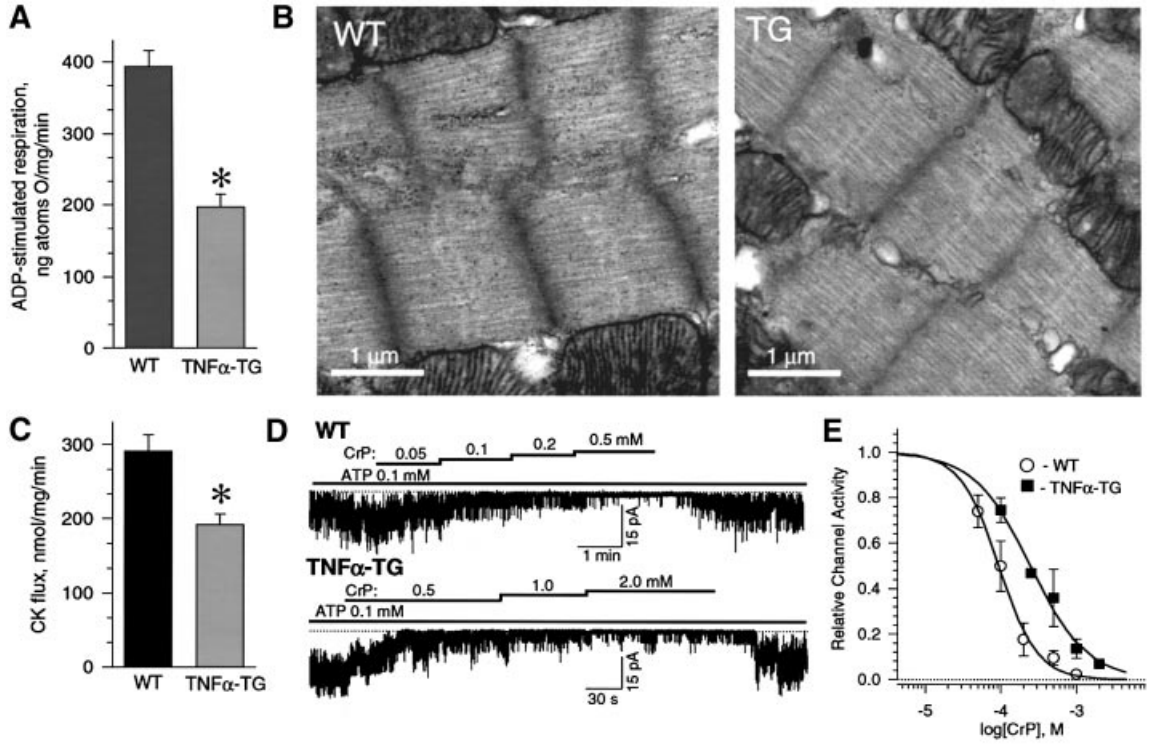

Fig. 3. Depressed bioenergetic components create conditions impeding signaling to K_{ATP} channels in TNFα-TG hearts. (**A**) Mitochondrial ADP-stimulated respiration is significantly depressed in isolated mitochondria from TNFα-TG compared with WT hearts. (**B**) While in WT glycogen granules are abundant in electron micrography, in TNFα-TG they are sparse. (**C**) Creatine kinase (CK) flux, by ^{18}O-assisted NMR spectroscopy, was significantly reduced in TNFα-TG compared with WT hearts. (**D**) In the open cell-attached mode, the creatine phosphate (CrP)/creatine kinase system effectively regulated K_{ATP} channel activity in the WT (upper), but not TNFα-TG (lower). (**E**) Concentration-response of CrP-stimulated K_{ATP} channel inhibition in open cell-attached patches. Data fitted by the Hill equation (solid curves) show a significant increase in the IC_{50} for CrP-induced channel inhibition in TNFα-TG versus WT. The asterisk in (A) and (C) indicates $p < 0.05$.

tolerated sympathetic challenge (n = 6), while all TNFα-TG cardiac cells (n = 6) developed intracellular calcium overload, precipitating contracture and ultimately cell death (Figure 5A). Morever, in animals stressed with isoproterenol (0.8–2.0 mg i.p.), contraction bands, a result of calcium overload (Karch and Billingham, 1986), were rare in WT (50 ± 11/heart), but were four times more frequent in the TNFα-TG (192 ± 20/heart) myocardium (n = 5, $p < 0.05$; Figure 5B, upper), seen on electron microscopy as sarcomere shortening (Figure 5B, lower). Potassium channel-opening drugs increase K_{ATP} channel activity through binding to channel proteins (Schwanstecher *et al*., 1998; Hambrock *et al*., 1999; Ashcroft and Gribble, 2000; Moreau *et al*., 2000). The clinically available potassium channel opener nicorandil activated K_{ATP} channels in membrane patches from failing hearts (n = 3; Figure 5C), and reversibly shortened action potential duration in isolated TNFα-TG heart (Figure 5D). Treatment of TNFα-TG animals with nicorandil (n = 4), as well as the structurally distinct K_{ATP} channel opener pinacidil (n = 3), reduced isoproterenol-induced contraction bands to a level comparable to the WT (Figure 5E), and improved energetic reserve assessed by storage of glycogen (Figure 5F). Thus, in failing hearts, improved tolerance to stress afforded by potassium channel openers underscores the contribution of K_{ATP} channel dysregulation to increased myocyte vulnerability in a disease environment.

Discussion

The ability of organisms to effectively respond to stress is crucial for health and survival, with an emerging recognition of the role of K_{ATP} channels in execution of this process (Zingman *et al*., 2003). While altered ATP responsiveness of K_{ATP} channels in hypertophied cardiomyocytes has been reported (Cameron *et al*., 1988; Yuan *et al*., 1997), it has remained unclear how disease states with consequent cellular remodeling affect the homeostatic function of K_{ATP} channels. Here, in a transgenic heart failure model, dysregulation of the K_{ATP} channel disrupted stress tolerance. This dysregulation was not due to alterations in the intrinsic biophysical properties of the channel, but rather to aberrant metabolic signaling to the channel preventing translation of distress under metabolic challenge into a protective membrane response. Disruption of this critical homeostatic mechanism exposes cells to injury in the disease environment of continuous stress confrontation, expanding the risk of disease progression.

The mitigated K_{ATP} channel response to cellular metabolic insult, despite intact basic properties of the channel, implicates alterations proximal to the channel, i.e. in ATP production and/or in transmission of energetic signals to the channel site, as the source of channel dysregulation. Indeed, the reduced potential for ATP production observed here is consistent with similar findings in human or experimental heart failure (Sharov *et al*., 2000; Liu *et al*., 2001), and TNFα-induced damage

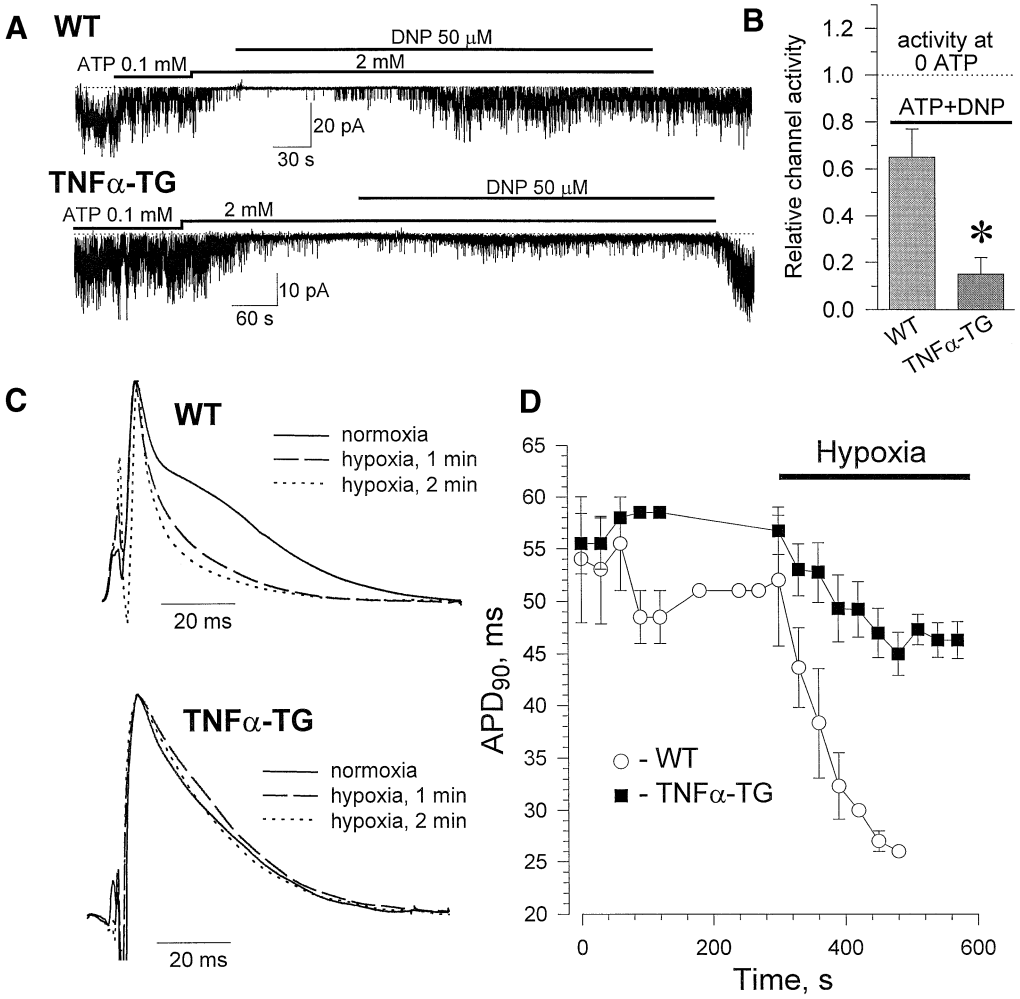

Fig. 4. K_{ATP} channel-dependent membrane control under stress defective in TNFα-TG hearts. (**A**) In the open cell-attached mode, with spontaneous channel opening suppressed by ATP, dinitrophenol (DNP) induced a vigorous K_{ATP} channel response in WT, but not TNFα-TG. (**B**) DNP-induced K_{ATP} channel activity in WT versus TNFα-TG ($p < 0.05$). (**C**) Monophasic action potentials under normoxia (O_2 content = 32 mg/l) and hypoxia (O_2 content = 3.1 mg/l). (**D**) Under hypoxia, APD_{90} markedly shortened in WT, but not TNFα-TG hearts.

to mitochondria through impaired mitochondrial DNA repair (Li *et al*., 2001). Normally, the majority of ATP is conveyed by creatine kinase to K_{ATP} channels, thereby overcoming time delays, concentration gradients and filtering effects of passive diffusion (Abraham *et al*., 2002). Modifications of this norm through remodeling in heart failure can occur by several mechanisms. As is shown here, and as has been established in general, flux or activity of creatine kinase is significantly diminished in failing hearts (Nascimben *et al*., 1996; Dzeja *et al*., 1999a). In addition, architectural alterations in the myocyte (this study; see also Chien, 1999; Hein *et al*., 2000; Bradham *et al*., 2002) could contribute to physical disruption of the phosphotransfer network and alter creatine kinase-dependent gating of K_{ATP} channels. These structural changes in failing myocytes would also heighten barriers to nucleotide diffusion and influence direct energetic crosstalk between cellular compartments (Kaasik *et al*., 2001; Sasaki *et al*., 2001), impeding K_{ATP} channel responsiveness to ATP. In fact, previous works demonstrate the dependence of proper K_{ATP} channel gating on the structural integrity of the myocyte (Brady *et al*., 1996; Furukawa *et al*., 1996; Terzic and Kurachi, 1996). Whether single or multiple alterations underlie defective signaling in heart failure, the inability of the creatine kinase system to properly regulate channel function relinquishes channel control to less efficient energy transfer, compromising the fidelity of the K_{ATP} channel response to cellular metabolic fluctuations under stress.

In heart failure, myocytes confront the metabolic insults of hypoxia, ischemia and adrenergic toxicity (Chien, 1999; Braunwald and Bristow, 2000; Bradham *et al*., 2002; Towbin and Bowles, 2002). Normal myocytes respond to such stressors by early activation of K_{ATP} channel-mediated K^+ conductance, resulting in action potential duration shortening, limitation of Ca^{2+} entry and myocardial protection (Nichols and Lederer, 1991). However, strikingly similar to the case of genetic deletion of K_{ATP} channels (Suzuki *et al*., 2002; Zingman *et al*., 2002a; Seino and Miki, 2003), the membrane response in TNFα-TG hearts to metabolic stress was characterized by a deficit in K_{ATP} channel opening and resultant action potential shortening. Accordingly, specific K_{ATP} channel blockers are unable to alter membrane repolarization in

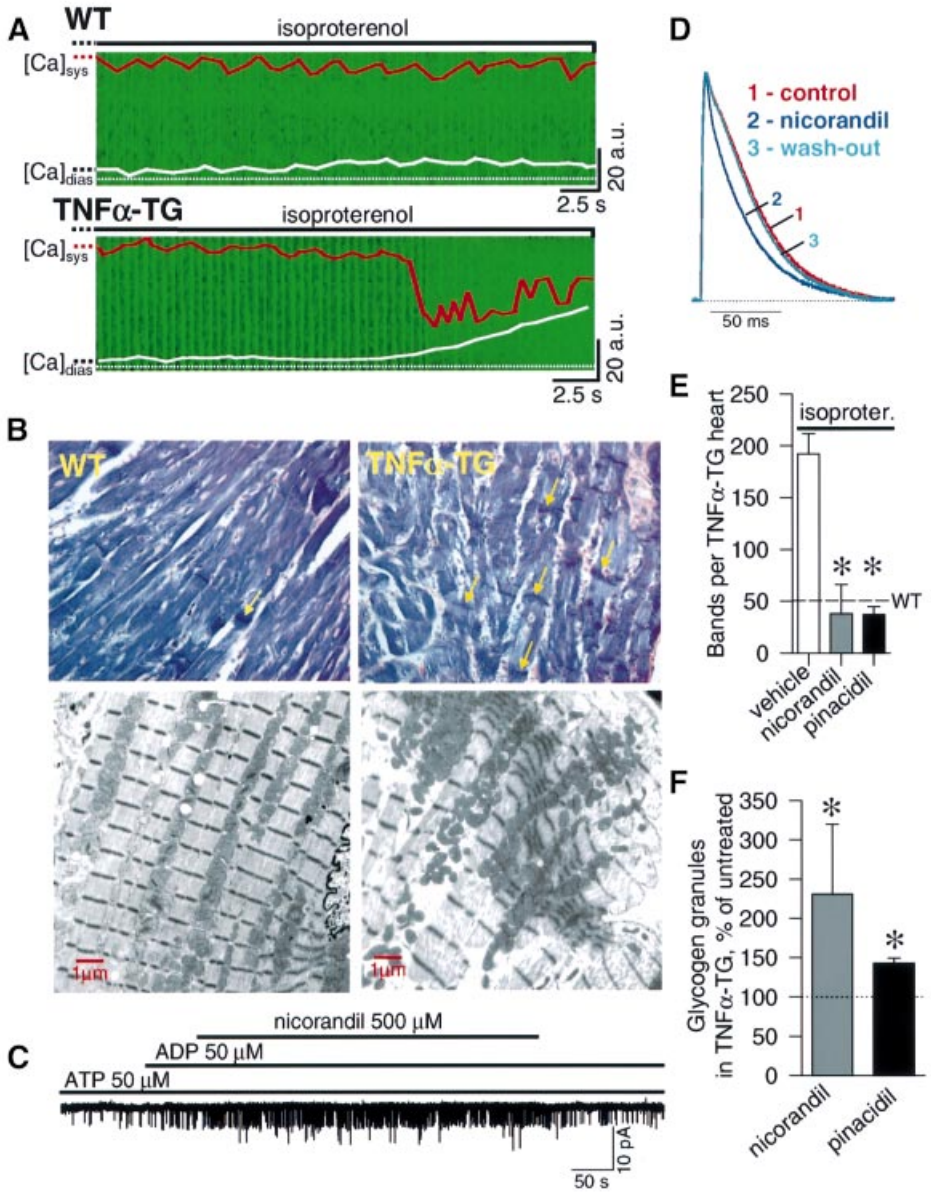

Fig. 5. Vulnerability to stress in TNFα-TG hearts attenuated by potassium channel openers. (**A**) Fluo-3-loaded and paced WT cardiomyocytes (upper) tolerated isoproterenol (1 μM) challenge without significant change in maximal systolic and diastolic Ca^{2+} levels. TNFα-TG cardiomyocytes (lower), under isoproterenol stress, developed diastolic Ca^{2+} overload with cell contracture. Ca^{2+}-induced fluorescence in a transverse cellular plane versus time is shown in green. Orange and white traces are deconvoluted fluorescent frames, and represent average Ca^{2+} maxima (systole, sys) and minima (diastole, dias). (**B**) Upper: photomicrographs (40×) of phosphotungstic acid hematoxylin-stained left ventricle 45 min after isoproterenol (2 mg i.p.) with contraction bands (arrows) in TNFα-TG, but not WT. Lower: electron microscopy of a contraction band in TNFα-TG (right) compared with normal sarcomeric pattern in WT (left). (C and D) In TNFα-TG, nicorandil (500 μM) activated K_{ATP} channels in excised patches (**C**), and shortened action potential duration (**D**). (**E**) Nicorandil (2 mg i.p; $n = 4$) or pinacidil (0.1 mg i.p; $n = 3$) versus vehicle ($n = 5$), 30 s prior to isoproterenol (isoproter.) challenge (0.8–2 mg i.p.), significantly reduced contraction bands in TNFα-TG mice ($p < 0.05$). Dotted line: average bands in vehicle-treated, isoproterenol-stressed, WT ($n = 3$). (**F**) Treatment of TNFα-TG mice twice daily for 1 week with nicorandil (0.5 mg/kg, s.c.; $n = 3$) or pinacidil (1 mg/kg, s.c.; n = 3) improved glycogen storage ($p < 0.05$) expressed relative to vehicle-treated TNFα-TG mice ($n = 3$; dotted line).

stressed failing hearts (Saavedra *et al.*, 2002). Thus, dramatically blunted and/or delayed membrane response to metabolic challenge provides a mechanistic basis for vulnerability to stress of cardiomyocytes in failing heart due to K_{ATP} channel dysregulation.

In contrast to K_{ATP} channel knockout hearts, failing TNFα-TG hearts retain intact intrinsic K_{ATP} channel properties, and thereby the potential for channel manipulation. The clinically used potassium channel opener nicorandil, in combination with mitochondrial preservation and nitrate-like effects, significantly activates sarcolemmal K_{ATP} channels in metabolically challenged cardiomyocytes (Shen *et al.*, 1991; Jahangir *et al.*, 1994), resulting in improved tolerance to insult (Tsuchida *et al.*, 2002). Here, in failing TNFα-TG hearts, nicorandil shortened action potential duration, attenuated calcium overload-associated contraction banding under stress, and improved glycogen stores. This is in line with

recent clinical studies in which cardioprotection conferred by nicorandil therapy was demonstrated in patients with ischemic heart disease (Patel *et al*., 1999; The IONA Study Group, 2002). The beneficial action of potassium channel openers in TNFα-TG failing hearts was further verified by pinacidil, a structurally distinct class member. Thus, potassium channel openers, in addition to their protective effect in cardiac ischemia, may have a role in attenuating myocardial injury in the setting of heart failure.

In summary, since their discovery two decades ago, K_{ATP} channels have been recognized as metabolic mediators with recent emphasis on their role in the adaptive response to stress. However, in disease states in which cells are remodeled under altered environmental demands, understanding of K_{ATP} channel behavior has thus far been limited. Here, in transgenic cytokine-induced heart failure, key features of human disease were recapitulated, including typical structural and energetic pathology. This remodeling, without influencing intrinsic K_{ATP} channel properties, translated into deficits in the potential for production and transmission of metabolic signals, thus compromising recognition of stress and adequate membrane homeostatic response. As continual cellular confrontation of stressors is characteristic of heart failure, loss of protective mechanisms expands the risk of disease progression. Thus, K_{ATP} channel metabolic dysregulation created by the disease state is a contributor to dysfunction and vulnerability in heart failure, illustrating a mechanism for acquired channelopathy in the absence of channel mutations. As deficits are proximal to channel proteins, the retained intrinsic K_{ATP} channel properties offer a therapeutic target for improved cell tolerance in disease.

Materials and methods

Transgenic mice

Heart failure was induced by cardiac-restricted overexpression of the cytokine TNFα using the α-myosin heavy chain promoter linked to the TNFα transgene (Sivasubramanian *et al*., 2001). Wild-type females were bred with transgenic males, and resultant heterozygous transgenic offspring (TNFα-TG), identified by tail-cut PCR, compared with WT littermates. Protocols were approved by the Institutional Animal Care and Use Committee at the Mayo Clinic.

Treadmill

TNFα-TG and WT mice were simultaneously exercised with increases in incline or velocity at 3 min intervals, on a two-track treadmill with a rear shock grid to enforce running (Columbus Instruments, Columbus, OH). Failure to exercise despite five shocks in 1 min defined time of drop-out. Tolerated workload (J) is the sum of kinetic ($E_k = m{\cdot}v^2/2$) and potential ($E_p = m{\cdot}g{\cdot}v{\cdot}t{\cdot}\sin\varphi$) energy, where m is animal mass, v is running velocity, g is acceleration due to gravity, t is elapsed time at a protocol level and φ is angle of incline.

Echocardiography

Two-dimensional M-mode echocardiographic images (Vingmed System FiVe; GE Medical Systems, Milwaukee, WI) in isoflurane-anesthetized mice were obtained at the mid-left ventricle from the parasternal long-axis view using a 10 MHz probe and a 2 cm gel stand-off (Parker Laboratories, Inc., Fairfield, NJ). Fractional shortening (%FS) was calculated as $\%FS = [(S - D)/D \times 100]$, where S is the end-systolic and D is the end-diastolic left ventricular chamber dimension (in cm) calculated using the leading-edge convention of the American Society of Echocardiography.

Isolated cardiomyocytes

The aorta was cannulated *in situ*, heart rapidly excised and retrogradely perfused at 90 mmHg for 5 min with HEPES buffer (Medium 199; Sigma), 1 min with a 'low-calcium' medium (100 mM NaCl, 10 mM KCl, 1.2 mM KH_2PO_4, 5 mM $MgSO_4$, 20 mM glucose, 50 mM taurine, 10 mM HEPES) supplemented with 0.13 mM $CaCl_2$, 2.1 mM EGTA, then 13 min with 'low-calcium' medium supplemented with 1% BSA, 0.2 mM $CaCl_2$, collagenase (type IV, 22 U/ml; Worthington) and pronase (100 μg/ml; Serva). Perfusion solutions were bubbled with 100% O_2. Ventricles were removed, cut into pieces (~3 × 3 mm) and incubated at 37°C for 15 min in the enzyme solution with gentle stirring. To harvest dissociated cardiomyocytes, supernatant was centrifuged at 500 r.p.m. for 1 min. The pellet was washed in 'low-calcium' medium supplemented with 0.2 mM $CaCl_2$ ('wash'), and again centrifuged. Finally, the pellet was suspended in 'wash' and kept at room temperature for 0–2 h until use. The harvest procedure was repeated three to five times on the incubated ventricle pieces to maximize yield. All solutions were at pH 7.25.

Microscopy

Paraffin sections of myocardium, stained with hematoxylin/eosin or Mallory's phosphotungstic acid hematoxylin, were examined by light microscopy. For field-emission scanning electron microscopy, isolated cardiomyocytes were fixed in PBS containing 1% glutaraldehyde and 4% formaldehyde (pH 7.2). Cells were dehydrated with ethanol and dried in a critical point dryer, coated with platinum using an Ion Tech indirect argon ion-beam sputtering system (VCR Group, San Francisco, CA), operating at accelerating voltages of 9.5 kV and 4.2 mA, and examined on a Hitachi 4700 field-emission scanning microscope (Perez-Terzic *et al*., 2001). For transmitted scanning electron microscopy, cells were post-fixed in phosphate-buffered 1% OsO_4, stained *en bloc* with 2% uranyl acetate, dehydrated in ethanol and propylene oxide, and embedded in low-viscosity epoxy resin. Thin (90 nm) sections were cut on an ultramicrotome (Reichert Ultracut E), placed on 200 μm mesh copper grids and stained with lead citrate. For glycogen analysis, tissue sections were processed with 2% uranyl acetate simultaneous with lead citrate. Micrographs were taken on a JEOL 1200 EXII electron microscope operating at 60 kV.

Patch–clamp electrophysiology

Isolated ventricular cadiomyocytes (~70 μm long) were bathed at 30 ± 1°C in 140 mM KCl, 10 mM HEPES, 5 mM glucose, 1 mM malate, 5 mM pyruvate, 5 mM EGTA and 1 mM $MgCl_2$ pH 7.4, and patched in the 'cell-attached' followed by the 'open cell-attached' or excised 'inside-out' mode using 5–10 MΩ pipettes filled with 140 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM glucose, at –60 mV. Following seal formation with the patch pipette in the cell-attached mode, the inside-out mode was obtained by patch excision whereas the open cell-attached mode was created by remote cell membrane permeabilization using digitonin (5–8 μg/ml) applied to the cell surface by a superfusion pipette (filled with 5 μg/ml propidium iodide and 0.5 μg/ml rhodamine). Formation of the open cell-attached patch configuration was indicated, under ultraviolet light, by propidium iodide staining of the cell nucleus, while rhodamine was used to indicate superfusion flow (Abraham *et al*. 2002). Single channel kinetics were analyzed within bursts of channel opening (Alekseev *et al*., 1998).

Mitochondria

Ventricles rapidly excised from anesthetized mice were removed into an ice-cold buffer composed of 50 mM sucrose, 200 mM mannitol, 5 mM KH_2PO_4, 1 mM EGTA, 5 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) pH 7.3, with 0.2% BSA. Tissue was homogenized (PT 10/35 Polytron; Brinkman Instruments, Westbury, NY), and the mitochondrial fraction obtained by differential centrifugation (Sorvall RCSC; Kendro Laboratory Products, Newtown, CT). Mitochondria were washed, suspended in isolation buffer (without EGTA and BSA) and kept on ice. Mitochondrial protein concentration was determined with a DC protein kit (Bio-Rad, Hercules, CA). Mitochondrial oxygen consumption was monitored with an oxygen-sensitive electrode, and data processed with the use of Bioquest software. State 3 (V_3) respiration was determined in the presence of 500 μM ADP (Ozcan *et al*., 2002).

Nuclear magnetic resonance

The aorta was cannulated *in situ*, heart excised and labeled for 30 s by perfusion with Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM Na-EDTA, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11 mM glucose, 1 mM malate and 5 mM pyruvate), containing 30% [^{18}O]water (Isotec), freeze-clamped, pulverized under liquid N_2 and then extracted in a solution containing 0.6 mM $HClO_4$ and 1 mM EDTA (Pucar *et al*., 2001). Protein content was determined with a DC Protein Assay kit (Bio-Rad). High-resolution ^{31}P NMR spectra of

tissue extracts were recorded on an Bruker 11 T spectrometer (Avance) at 202.5 MHz. Peak integrals were determined with a built-in integration routine (Xwinnmr 2.5 software; Bruker). Creatine kinase phosphotransfer rate was determined from the appearance rate of ^{18}O-labeled creatine phosphate species using pseudo-linear approximation, and ATP levels determined using methylene diphosphonic acid as standard (Pucar *et al*., 2001).

Monophasic action potentials

The aorta was cannulated *in situ*, heart excised and retrogradely perfused at 90 mmHg with Krebs–Henseleit buffer filtered at 0.22 μm and bubbled with 95% O_2/5% CO_2 at 37°C and pH 7.4. Hearts were paced (A310 Accupulser; World Precision Instruments, Sarasota, FL) by a catheter positioned in the right ventricular apex (NuMed, Hopkinton, NY). A monophasic action potential probe (EP Technologies, San Jose, CA) was maintained in a single position on the left ventricular epicardium, and the amplified signals (IsoDam; World Precision Instruments) acquired at 11.8 kHz. For hypoxia, the perfusate was bubbled with 90% N_2/5% O_2/ 5% CO_2.

Calcium imaging

Scanning confocal images (256 × 256 pixels) of isolated cardiomyocytes, loaded with Fluo3-AM (3 μM; Molecular Probes) and paced at 2 Hz, were acquired every 328 ms using the 488 nm line of an argon/krypton laser. Images were analyzed by MetaMorph software (Visitron Universal Imaging, Downingtown, PA).

Statistics

Data are expressed as mean ± SE. Comparisons are by paired Student's *t*-test or analysis of variance. Survival is presented as the Kaplan–Meier product-limit estimate from which killed mice were censored, and differences determined by log-rank test. A p value <0.05 is considered significant.

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