Chronic type 2 but not type 1 diabetes impairs myocardial ischaemic tolerance and preconditioning in C57Bl/6 mice

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New Findings

What is the central question of this study?
Influences of types 1 and 2 diabetes on myocardial ischaemic tolerance and cardioprotection are contentious. Opposing outcomes across models may reflect importance of disease duration and onset age. Chronic adult onset diabetes was modelled non-genetically in C57Bl/6 mice to assess impacts on cardiac ischaemic outcomes and preconditioning.

What is the main finding and its importance?
The primary finding is that chronic adult-onset type 2 but not type 1 diabetes significantly impairs myocardial ischaemic tolerance and ischaemic preconditioning. Preconditioning may be detrimental in type 2 diabetes, exaggerating nitrosative stress and apoptotic protein expression.
Abstract

Effects of diabetes on myocardial responses to ischaemia-reperfusion (I-R) and cardioprotective stimuli remain contentious, potentially reflecting influences of disease duration and time of onset. Chronic adult-onset type 1 diabetes (T1D) and 2 diabetes (T2D) were modelled non-genetically in male C57Bl/6 mice via 5 x 50 mg/kg daily streptozotocin (STZ) injections + 12 week standard chow; or 1 x 75 mg/kg STZ injection + 12 weeks obesogenic diet (32% calories as fat, 57% carbohydrate, 11% protein), respectively. Systemic outcomes were assessed and myocardial responses to I-R ± ischaemic preconditioning (IPC; 3 x 5 min I-R) determined in Langendorff perfused hearts. Uncontrolled T1D was characterised by pronounced hyperglycaemia (25 mM fasting glucose), glucose intolerance and ~10% body weight loss, whereas T2D mice exhibited moderate hyperglycaemia (15 mM), hyperinsulinemia, glucose intolerance and 17% weight gain. Circulating ghrelin, resistin and noradrenaline were unchanged with T1D, while leptin increased and noradrenaline declined in T2D mice. Ischaemic tolerance and IPC were preserved in T1D hearts. In contrast, T2D worsened post-ischaemic function (~40% greater diastolic and contractile dysfunction) and cell death (100% higher troponin efflux), and abolished IPC protection. Whereas IPC reduced post-ischaemic nitrotyrosine and pro-apoptotic Bak and Bax levels in non-diabetic hearts, these effects were reduced in T1D and IPC augmented Bax and nitrosylation in T2D hearts. Data demonstrate chronic T1D does not inhibit myocardial I-R tolerance or IPC, whereas metabolic and endocrine disruption in T2D is associated with ischaemic intolerance and inhibition of IPC. Indeed, normally protective IPC may exaggerate damage mechanisms in T2D hearts.
**Abbreviations:**

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<td>EDP</td>
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Introduction

Ischaemic heart disease and myocardial infarction pose major health challenges worldwide. An important contributor is increasingly prevalent type 2 diabetes (T2D), which increases cardiovascular disease risk up to 5-fold (Rivellese, Riccardi, & Vaccaro, 2010). More controversially, diabetes may also worsen infarct damage and inhibit protective signalling and responses to ‘conditioning’ interventions (Miki, Itoh, Sunaga, & Miura, 2012). However, conflicting findings emerge from clinical and experimental studies of the influences of diabetes on ischaemic tolerance and cardioprotection. There is some evidence myocardium from those with T1D or T2D is less resistant to I-R injury, including exaggerated oncotic and apoptotic death (Chowdhry, Vohra, & Galinanes, 2007) and post-ischaemic contractile dysfunction (Hoogslag et al., 2015). Hyperglycaemia alone may also increase cardiac injury and mortality in patients with myocardial infarction (Capes, Hunt, Malmberg, & Gerstein, 2000). Other studies report no difference in myocardial damage between STEMI patients ± diabetes (Reinstadler et al., 2017), and ischaemic tolerance in models of T1D/hyperglycaemia ranges from unaltered (Ebel et al., 2003; Ghaboura et al., 2011) to reduced (Di Filippo et al., 2005; Liu, Wei, Peng, Layne, & Yet, 2005) or improved (Liu, Thornton, Cohen, Downey, & Schaffer, 1993; Pourkhalili et al., 2012; Tosaki, Engelman, Engelman, & Das, 1996). There are also reports of unaltered (Ghaboura et al., 2011; Przyklenk, Maynard, Greiner, & Whittaker, 2011) or worsened I-R tolerance (Bouhidel et al., 2008; Chen et al., 2016; Katakam et al., 2007; Miki et al., 2009) in models of T2D. Conflicting observations similarly emerge regarding cardioprotective signalling and responses (Liu et al., 1993; Rana & Sharma, 2016). While there is evidence of impaired preconditioning in both in vivo and ex vivo myocardium (Ghosh, Standen, & Galinianes, 2001) from diabetics, and of declining efficacies of GPCR and mitochondria targeted interventions (Hassouna et al., 2006), some studies report preservation of cardioprotection in
diabetes (Liu et al., 1993; Peake et al., 2013; Rana & Sharma, 2016). These conflicting observations may reflect important influences of the time of disease onset and its duration (particularly critical to tissue stress-resistance) together with limitations in different models of disease. Distinct changes may emerge in transition to chronic disease, since acute and low-grade stressors can induce adaptation and protection whereas chronic stress has the opposite effect (McEwen, 2004). Tissue protection vs. injury may thus emerge with acute vs. chronic ischaemic, hypoxic or oxidative stressors and protein O-GlcNAcylation, for example. Apparently sustained I-R tolerance following neonatal induction of T1D (Liu et al., 1993) reveals the additional importance of timing of disease onset: early life vs. adult stressors induce distinct effects, with early developmental plasticity facilitating adaptation to later life (Bateson, Gluckman, & Hanson, 2014). This highlights potential limitations in inbred disease-prone lines (e.g. Goto-Kakizaki rats) and genetic models (e.g. \(db/db\) mice), which involve disease development from very early life. For example, obesity and hyperglycaemia arise prior to maturation in \(db/db\) mice, with hyperinsulinemia and abnormalities in myocardial metabolism, MVO\(_2\) and efficiency emerging even earlier (Buchanan et al., 2005). Another potential drawback is intrauterine programming of stress phenotype, with maternal hyperglycaemia inducing myocardial inflammation and oxidative stress in early neonates (Higa et al., 2017) and I-R intolerance in adult offspring (Gao et al., 2016). Early disease onset and possible maternal influences may thus limit the utility of such models in delineating effects of adult disease on myocardial stress-resistance: T2D predominantly emerges from early adulthood on (with 4-6 yrs from metabolic dysfunction to T2D diagnosis) (Porta et al., 2014), and late onset or latent autoimmune diabetes of the adult is even more prevalent than childhood T1D (Laugesen, Ostergaard, Leslie, Danish Diabetes Academy, & Workshop, 2015).

To better understand the myocardial impacts of chronic disease commencing in early
adulthood, we study non-genetic T1D/T2D induction in young adult mice with progression over a 12 week period, corresponding to ~10 human yrs (the period from metabolic dysregulation to diagnosis in humans (Porta et al., 2014) or ~2 yrs of biological time (based on 7-fold higher murine metabolic rate). For T2D we employ a combination of Islet cell stress and obesogenic diet, while T1D involves uncontrolled hyperglycaemia as a result of Islet cell destruction. Cardiac, metabolic and endocrine changes were assessed, with a focus on intrinsic I-R tolerance and responses to the prototypic protective stimulus IPC.

Methods

Ethical approval All investigations were approved in accordance with policy guidelines (The Animal Care and Protection Act 2001) of the Animal Ethics Committee of Griffith University (ethics approval MSC/14/16/AEC), which is accredited by the Queensland Government, Australia. Male C57Bl/6 mice were supplied by the Animal Resource Centre (Perth, Australia) and housed in the Griffith University Animal Facility for the duration of the study. Mice were habituated to the facility for at least 1 week prior to studies and were housed in groups of 4, with sawdust bedding and ad lib access to water and food throughout. The mice were maintained in a 12-hour day/night lighting cycle at a constant temperature of 21°C and 40% humidity. Sodium pentobarbital (60 mg/kg, intraperitoneal) was used to euthanize mice at the end of the study. Methods and experiments complied with the ethical regulations and principles of Experimental Physiology (Grundy, 2015) and ensured animal pain and suffering was minimised.

Murine diabetes models To model chronic T1D, 8 week old male C57Bl/6 mice were administered 5 x daily intraperitoneal (IP) injections of STZ (50 mg/kg) and were maintained on standard chow for 12 weeks (n=18). To model chronic T2D (n=18), 8 week male C57Bl/6
mice were administered STZ (75 mg/kg IP) and maintained on a high-saturated fat/high-sugar chow for 12 weeks (31.9% calories as fat, 56.7% as carbohydrates and 11.3% as protein). Obesogenic chow was freshly formulated weekly, consisting of 800 g Irradiated Rat & Mouse Powder (Specialty Feeds; Glen Forrest, WA, Australia), 300 g Nestle condensed milk, 140 g refined sugar and 125 g animal fat (Supafry blended edible animal fat solidified oil). Non-diabetic control mice were administered Na-citrate injections and maintained on standard chow for 12 weeks (CTRL n=29). Food and water were available ad libitum for all groups.

**Biochemical assessment** Glucose tolerance tests (GTT) were performed at 10 weeks of diabetes development (post-STZ challenge). Mice were fasted for 4-6 hrs before blood was taken via tail prick for measurement of fasting blood glucose (Accu-Check II glucometer; Roche Diagnostics, Castle Hill, Australia). Mice then received IP administration of 20% glucose solution (2 g glucose/kg) with blood levels assayed at 30 min intervals over 3 hrs. GTT AUC was calculated to compare clearances between control and type 1 or type 2 diabetic mice.

For assessment of neuroendocrine changes, blood was collected at sacrifice into EDTA (serum) or heparin (plasma) coated tubes. Whole blood was incubated at room temperature for 30 min before 5 min centrifugation at 10,000 g, with serum samples removed and stored at -80°C until a randomly selected subset of serum analysed via ELISA according to manufacturer’s instructions (insulin: Crystal Chem Inc, Elk Grove Village, USA; ghrelin, leptin and resistin: Cusabio, Houston, Texas, USA; noradrenaline: Abnova, Taipei City, Taiwan), using a Tecan plate reader (diabetics, n=6-7; CTRL, n=13).

**Cardiac perfusion and I-R responses** At the end of experimental periods hearts were
removed and Langendorff perfused (Headrick, Peart, Hack, Flood, & Matherne, 2001; Reichelt, Willems, Hack, Peart, & Headrick, 2009) for assessment of function, I-R tolerance \((n=10\) for T1D and T2D, \(n=21\) for CTR) and efficacy of IPC \((n=8\) for all groups). Mice were anaesthetised with sodium pentobarbital \((60\ \text{mg/kg IP})\), hearts excised and the aorta cannulated for perfusion of the coronary circulation with modified Krebs-Henseleit buffer, gassed with 95% O\(_2\)/5% CO\(_2\), maintained at 37°C \((\text{pH 7.4})\) and containing: 119 mM NaCl, 11 mM glucose, 22 mM NaHCO\(_3\), 4.7 mM KCl, 1.2 mM MgCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM EDTA, 0.5 mM and 2.5 mM CaCl\(_2\).

Contractile function was monitored via fluid-filled balloon in the left ventricle, inflated to an end-diastolic pressure \((\text{EDP})\) of 5 mmHg (Headrick et al., 2001; Reichelt et al., 2009). Coronary flow was measured via ultrasonic flow-probe proximal to the aortic cannula and connected to a T206 flowmeter (Transonic Systems Inc., Ithaca, NY, USA). A 4 channel MacLab system (ADInstruments Pty Ltd., Castle Hill, Australia) coupled to an Apple iMac computer was used for acquisition \((1\ \text{KHz sampling rate})\) and processing of data, including: systolic and end-diastolic pressures, \(+dP/dt\) and \(-dP/dt\), heart rate and coronary flow. Temperature of perfusate was continuously monitored via thermal probe connected to a Physitemp TH-8 digital thermometer (Physitemp Instruments Inc, Clifton, NJ, USA).

Hearts were stabilised over 20 min, with any exhibiting abnormal or unstable function excluded, as detailed previously (Reichelt et al., 2009). Hearts were then switched to ventricular pacing at 420 beats/min \((\text{via silver wires attached to an SD9 stimulator; Grass Instruments, Quincy, MA, USA})\) for 10 min before induction of 25 min normothermic global ischaemia followed by 45 min aerobic reperfusion. Based on our prior work (Headrick et al., 2001; Peart & Headrick, 2003; Reichelt et al., 2009), such an insult is predicted to induce significant cell death and ~50% depression of contractile function. For IPC, hearts were subjected to 3 x 5 min episodes of ischaemia separated by 5 min reperfusion, a stimulus
substantially greater than the 3 x 1.5 min algorithm protecting hearts of healthy mice (J. N. Peart et al., 2014) and shown to overcome the inhibitory effects of diabetes (albeit in rats) (Tsang, Hausenloy, Mocanu, Carr, & Yellon, 2005). To estimate myocardial death, efflux of cardiac troponin I (TnI) was measured in randomly selected subsets of hearts: total post-ischaemic coronary effluent was collected on ice and TnI content assayed via ELISA (Life Diagnostics; West Chester, PA, USA) (n=6-8/group), with efflux normalised to heart mass. We confirm strong linear correlation between protein efflux and infarct size in this model (Peart & Headrick, 2003), with TnI specific to myocytes and robustly correlating with infarction in humans (Hallen, 2012).

**Myocardial tissue analysis** Ventricular lysates from a randomly selected subset of post-ischaemic hearts were assayed for protein expression (n=5-8/group) and nitrotyrosine (diabetics, n=7-9; CTRL non-IPC, n=14; CTRL IPC, n=7) content. Tissue was homogenised in a glass dounce with 1.0 mL of ice-cold lysis buffer containing 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 20 mM NaPP and 1% Triton X-100, together with protease and phosphatase inhibitors: 1 mM PMSF, 10 μM leupeptin, 3 mM benzamidine, 5 μM pepstatin A, 1 mM sodium orthovanadate. Total protein content was assayed via Pierce™ BCA Protein assay (Thermo Fisher Scientific; Scoresby, Australia) and samples diluted to ~1 μg/μL protein. Aliquots were stored at -80°C until western immunoblot analysis or 3-nitrotyrosine content. The latter was assayed via ELISA (Abcam cat. no. ab113848; Cambridge, UK), using a 3-nitrotyrosine-BSA standard curve to estimate sample 3-nitrotyrosine content, as per manufacturer’s instructions.

**Western immunoblotting** Lysate samples were mixed with equal volumes of 2x loading dye containing 5% β-mercaptoethanol and denatured at 95°C for 5 min, before cooling on ice.
A total of 30 μg protein/sample was loaded into hand-cast 10% acrylamide gels and separated via electrophoresis at 150 V for 60 min. Proteins were transferred to a polyvinylidene difluoride fluorescent (PVDF) membrane at a constant 75 V for 1.5 hrs, and blocked with Odyssey fish serum for 2 hrs at room temperature. Transferred proteins were incubated for 18 hrs at 4°C with gentle rocking in primary antibody, including: Bax (Cell Signalling #2772, 1:1000); Bak (Cell Signalling #D4E4, 1:1000); total-AKT (Cell Signalling #9272S, 1:1000); phosphorylated-AKT (Ser473, Cell Signalling #9271, 1:1000); and GAPDH (Santa Cruz sc-32233, 1:5000). Membranes were washed 4 times in TBST (5 min each) and again in TBS for 5 min before incubation with secondary antibodies (LI-COR IRDye® 680RD, donkey anti-mouse: cat. no. 925-68072; or goat anti-rabbit: cat. no. 925-68071, 1:30,000) at room temperature in the dark. Membranes were again washed 4 x 5 min in TBST and 5 min in TBS before drying overnight between paper towels in the dark. Membranes were visualised on a LI-COR Odyssey Infrared Imaging System (Millenium Science; Mulgrave, Australia) with protein densitometry data normalised to GAPDH in each sample.

**Statistical analysis** Specific *a priori* hypotheses tested include: i) T1D or T2D worsens I-R tolerance; and ii) T1D or T2D inhibits protection via IPC. An ANOVA with planned comparisons was employed to test these questions (does either model of diabetes worsen I-R tolerance; is IPC effective in T1D or T2D), eliminating nonsensical contrasts constraining statistical power (Ludbrook, 1991). Data were analysed using GraphPad Prism 7 and presented as means±SD. An ANOVA was performed followed by Fisher’s LSD post-hoc test for planned comparisons. A Student’s t-test was used for comparisons between two groups. A P-value <0.05 was indicative of statistical significance across tests.
Results

Systemic outcomes  Opposing effects on body weights were observed, with a significant 9% decline in T1D (~26 g) vs. CTRL (30 g), and 17% increase with T2D (to 35 g) (Figure 1A,B). Diabetic mice displayed significant fasting hyperglycaemia, an effect more profound in T1D than T2D mice (Figure 1C). The glucose tolerance test AUC followed a similar pattern, with significant glucose intolerance that was more extreme in T1D compared to T2D (Figure 1D). Insulin increased more than 2-fold in T2D vs. CTRL mice (Figure 1E). The Homeostasis Model Assessment (HOMA) ratio, calculated as the ratio of fasting insulin:glucose, was markedly elevated in T2D mice (Figure 1F). Leptin was significantly increased in T2D and reduced in T1D vs. CTRL mice (Figure 2), while ghrelin and resistin levels were unchanged. Circulating noradrenaline was reduced in T2D vs. CTRL mice (Figure 2).

Effects of T1D and T2D on myocardial I-R tolerance and IPC  Ischaemic contracture was differentially influenced by T1D and T2D, and by IPC (Figure 3). Peak ischaemic contracture was elevated in T2D vs. CTRL hearts, but not with T1D, with the rate of contracture development similar across groups. Treatment with IPC had no effect on peak contracture in CTRL or T2D hearts, while increasing degree of contracture in T1D hearts. Rate of contracture development was accelerated by IPC in CTRL and T1D hearts (Figure 3).

Final recovery of left ventricular developed pressure (LVDP) was unchanged in T1D vs. CTRL, but significantly reduced (~15%) in T2D vs. CTRL hearts (measured after 45 min reperfusion) (Figure 4). This effect of T2D primarily reflected exaggerated diastolic dysfunction, with recovery of systolic pressure only modestly repressed. Final post-ischaemic EDP was over 50% higher in T2D vs. CTRL hearts (Figure 4). Preconditioning significantly improved cardiac functional outcomes in CTRL and T1D but not T2D hearts, whereas recovery of coronary flow was selectively improved in T2D but not CTRL or T1D hearts.
Post-ischaemic TnI efflux was significantly higher in T2D vs. CTRL hearts, with an insignificant trend to reduced efflux in T1D hearts \( (p=0.20) \). Treatment with IPC reduced TnI in CTRL hearts only.

**Post-ischaemic apoptotic proteins, phospho-AKT and nitrosylation levels**

Post-ischaemic levels of pro-apoptotic Bax were reduced in both T1D and T2D hearts, with Bak also reduced in T1D hearts (Figure 5). Post-ischaemic Bax and Bak were reduced by IPC in CTRL but not T1D or T2D hearts. IPC significantly augmented Bax expression in the latter T2D group. Neither post-ischaemic expression nor phosphorylation of AKT differed in hearts from diabetic vs. healthy animals. A tendency to moderate phosphorylation was evident with IPC in CTRL and T1D (but not T2D) hearts, though this was not statistically significant. There were no differences in post-ischaemic nitrotyrosine in non-preconditioned hearts from CTRL and diabetic mice (Figure 5). Accumulation of nitrotyrosine was reduced by IPC in CTRL hearts, whereas this protective effect was lost in T1D hearts and appeared to be reversed to an increase (albeit not reaching statistical significance; \( p=0.10 \)) in T2D hearts (Figure 5).

**Correlates of myocardial I-R tolerance**

Post-ischaemic dysfunction and death appeared to correlate with ischaemic contracture in non-preconditioned hearts from healthy, T1D and T2D mice, suggesting some role for diastolic dysfunction in exaggerated cardiac injury with diabetes (Figure 6A). This correlation was abolished by IPC, likely reflecting an amalgam of known stimulatory effects of IPC on contracture coupled with induction of protective mechanisms. Conversely, nitrosylation specifically correlated with I-R outcomes in IPC hearts with this link not apparent in non-preconditioned hearts (Figure 6B). This suggests nitrosylation may play a
more significant role in determining the effects of IPC than intrinsic I-R tolerance.

Discussion

Data reveal that despite profound hyperglycaemia, 12 weeks of simulated T1D in adult male mice does not significantly impair cardiac I-R tolerance or protection via IPC. However, less severe hyperglycaemia in the context of T2D is associated with significantly worsened myocardial I-R injury, loss of protection via IPC and a potential reversal of its effects on injury mediators (increased Bax expression and cardiac nitrosylation). Chronic hyperglycaemia thus does not appear to be a primary determinant of I-R tolerance or IPC efficacy, unless coupled with insulin-resistance and additional endocrine/metabolic disruption in T2D. Although assessing outcomes in models of untreated diabetes (with a goal of identifying disease impacts independent of therapy), these observations suggest management of glucose levels alone may be insufficient to counter abnormalities in myocardial stress-resistance and cardioprotection.

Systemic features of T1D and T2D models

The two models exhibit distinct systemic outcomes, with weight loss vs. gain and more profound hyperglycaemia and glucose intolerance in T1D vs. T2D (Table 1). Disturbed neuroendocrine control was more evident in T2D mice, including elevated circulating leptin and reduced noradrenaline. Leptin reportedly mediates acute cardioprotection (Smith et al., 2010), whereas chronic elevations in obesity and diabetes may be detrimental and lead to/reflect leptin-resistance. Resistance will not only limit cardioprotection (Smith et al., 2010), but influence food intake and metabolism, inflammation, oxidative stress, apoptosis and tissue remodelling (Martin, Qasim, & Reilly, 2008). Tissue-specific leptin-resistance may emerge with age and metabolic disorders, though
there is evidence of better preservation of leptin sensitivity in the heart (Stucchi et al., 2011). Nonetheless, cardiovascular leptin-resistance does arise in obesity and diabetes, apparent in both coronary (Knudson et al., 2005) and myocardial cells (Ren, Zhu, Relling, Esberg, & Ceylan-Isik, 2008).

We also observe a small but significant decline in circulating noradrenaline, contrasting some studies in genetic models (Martín-Cordero, García, Hinchado, & Ortega, 2011) though consistent with Caviezel et al. (1982) and Peschke et al. (2011). The latter authors propose decreased noradrenaline is important in insulin-resistance and insulin-melatonin interactions in T2D. A fall in noradrenaline may also influence cardiac contractile and electrical activities and cardiomyopathy: diabetic autonomic neuropathy limits cardiac noradrenaline release and responses to load/stress, and myocyte responses to noradrenaline are impaired in diabetic cardiomyopathy. Interestingly, while myocardial noradrenaline release (Burgdorf et al., 2003) and responses (Galderisi et al., 2007) are inhibited in T2D, noradrenaline-dependent vasoconstriction is differentially augmented (Nguyen Dinh Cat et al., 2018), supporting distinct vascular vs. cardiac outcomes.

Metabolic and neuroendocrine distinctions between T1D and T2D may be relevant to differences in I-R tolerance and IPC. Insulin- and leptin-resistance in T2D may limit receptor-mediated protection and activation of distal survival kinases. Reduced noradrenaline levels (coupled with altered α/β-adrenoceptor expression) may also influence I-R tolerance, cardioprotection, and remodelling in addition to promoting hyperinsulinemia and insulin-resistance (Peschke et al., 2011).

Effects of T1D on myocardial I-R tolerance and IPC

There remains uncertainty regarding impacts of diabetes on myocardial I-R tolerance and cardioprotection (Miki et al., 2012). Some of this variance may well reflect distinct impacts
of acute vs. chronic and early life vs. adult onset disease (Bateson et al., 2014; McEwen, 2004). Myocardial outcomes in models of acute disease (e.g. 1-4 weeks hyperglycaemia) may not reflect the chronic disease phenotype, as suggested in rodent models of T1D: I-R tolerance transiently improves in the initial weeks of STZ- or alloxan-induced hyperglycaemia (Pourkhalili et al., 2012; Tosaki et al., 1996), may recover after 2-8 weeks (Chen et al., 2013; Garcia-Cardena et al., 1997; Ghaboura et al., 2011; Jamwal, Kumar, & Reddy, 2016; Lin et al., 2016; Potier et al., 2013; Przyklenk et al., 2011; Zhang et al., 2016), and decline with chronic (≥8 weeks) disease (Li et al., 2016; Liu et al., 2013; Xu, Takashi, Kudo, Ishiwata, & Naito, 2004; Xue et al., 2016). We assessed a 12 week period of disease progression in adult mice, observing no change in myocardial outcomes from a 25 min ischaemic insult that suppresses contractile function by ≥50% and induces significant cell death. Whether detrimental impacts of T1D might emerge with more severe I-R is unknown, however data clearly reveal a relative insensitivity of cardiac stress-resistance/IPC to T1D vs. T2D. Interestingly, data also reveal significant reductions in post-ischaemic Bax and Bak expression, suggestive of potential benefits with prolonged T1D, though this does not translate to improved function or cell survival.

Prior studies also report inhibition of protective responses in models of T1D, including pre- (Yadav, Singh, & Sharma, 2010) and post-conditioning (Li et al., 2016; Liu et al., 2013; Przyklenk et al., 2011). However, others observe preservation of IPC in T1D (Ghaboura et al., 2011; Liu et al., 1993). Our findings agree with the latter, revealing significant benefit via IPC in hearts from mice exhibiting chronic hyperglycaemia for 12 weeks. Effects of IPC were nonetheless modified in this model, which appeared to limit its impacts on cell death, nitrotyrosine, Bax and Bak.
Effects of T2D on myocardial I-R tolerance and IPC

In contrast to T1D, T2D substantially reduced I-R tolerance and eliminated protection via IPC (Figure 4). Ischaemic intolerance is consistent with some prior studies (Bouhidel et al., 2008; Chen et al., 2016; Katakam et al., 2007; Miki et al., 2009). The observed increase in TnI efflux despite reduced pro-apoptotic protein expression is suggestive of differential influences on oncotic vs. apoptotic death. While some observe increased apoptosis in models of T2D, other evidence suggests oncotic death is augmented to a greater extent (Das et al., 2015). Differences in stress-resistance and cardioprotection appear independent of post-ischaemic phospho-AKT levels (Figure 5), in agreement with several prior studies (Bouhidel et al., 2008; Desrois et al., 2004). Correlation of intrinsic I-R tolerance with ischaemic contracture also suggests a role for exaggerated diastolic dysfunction (characteristic of T2D) in worsened post-ischaemic outcomes.

Consistent with distinct cardiac impacts of T1D vs. T2D, Ansari et al. recently reported (in the course of our study) that I-R tolerance is impaired in hearts from rats fed a high-fat diet for 12 weeks with STZ injection at 8 weeks, yet not from rats subjected to STZ-dependent hyperglycaemia alone (Ansari, Gopalakrishnan, & Kurian, 2019), paralleling differing mitochondrial dysfunction. In the db/db genetic model of T2D, Wang et al. (2018) report reduced I-R tolerance, attributing this to a fall in miR-24, which protects healthy and db/db hearts when overexpressed (though whether normalising I-R tolerance across groups was not tested). Our findings also agree with those of Hjortbak et al. (2018) in the Zucker diabetic fatty rat model, in which infarct tolerance was reduced in 24 week old animals.

Insulin-resistance may be particularly important to impairment of I-R tolerance, as insulin receptors induce protection. We have shown that emergence of insulin-resistance is critical to the effects of obesity on I-R responses and cardioprotection. Jelenik et al. recently reported that insulin-resistance coupled with increased lipid availability may sensitise hearts...
to I-R injury (Jelenik et al., 2018). In terms of I-R tolerance, severity of hyperglycaemia per se may be less important than insulin-resistance, which not only impairs glucose transport but reduces activation of survival kinase pathways and maintenance of mitochondrial stability and function.

Additional to declining ischaemic tolerance, IPC was ineffective in T2D hearts, agreeing with reports of impaired pre- and post-conditioning (Przyklenk et al., 2011) in other models. On the other hand, a recent study reports preserved IPC in the Zucker diabetic fatty rat model at 6 (pre-diabetes), 12 (diabetes onset) and 24 weeks (established disease). The basis for this opposing outcome is unclear. IPC involves activation of multiple membrane receptors (including GPCRs and RTKs) and kinase signalling pathways. The observation that some protective stimuli retain efficacies in diabetes, including sphingosine-1 and hydrogen sulfide interventions (Bulhak et al., 2009; Peake et al., 2013; Rana & Sharma, 2016), indicates that some survival paths do remain intact in diabetic myocardium. Evidence that an increased amplitude of the IPC stimulus may overcome inhibitory effects in hearts from Goto-Kakizaki rats (Tsang et al., 2005), also highlights the possibility hearts are desensitised rather than completely resistant to protective stimuli (i.e. exhibiting higher cardioprotective 'thresholds'). However, questions arise regarding the relevance of the Goto-Kakizaki and similar models to adult-onset disease and its cardiac sequelae given possible maternal influences on tissue stress/I-R tolerance and lifelong metabolic and cardiac abnormalities in such animals. While we test an IPC stimulus >3-fold greater (based on ischaemic duration) than that protecting heathy hearts (Peart et al., 2014), and which Tsang et al. (2005) report overcomes inhibitory effect of diabetes on IPC in rats, we cannot exclude the possibility that an even more profound IPC stimulus might be protective in T2D hearts. Interestingly, IPC specifically improved recovery of coronary flow in T2D hearts, despite worsened contractile outcomes and cellular injury. Preconditioning has been shown to improve coronary vascular
outcomes in both non-diabetic and diabetic hearts (Bouchard & Lamontagne, 1998), however the basis of this selective vascular outcome in T2D but not control or T1D hearts is unclear.

Mechanisms by which IPC protects include suppression of nitrosative stress and of apoptotic proteins (Nakamura et al., 2000). An IPC-dependent reduction in nitrosylation in non-diabetic hearts is congruent with cardioprotection, whereas data suggest IPC may augment nitrosative stress in hearts from T2D mice (Figure 6). Myocardial nitrotyrosine levels are known to increase during an IPC stimulus while subsequent accumulation during prolonged I-R is reduced (Juhasz et al., 2011). This initial nitrosylation may contribute to protection, potentially reflecting differential modulation of nitrosative stress by nNOS (Lu et al., 2009). Interestingly, I-R outcomes appear consistently related to post-ischaemic nitrosylation in preconditioned yet not non-preconditioned hearts (Figure 6), congruent with a more prominent role for nitrosylation in IPC-dependent outcomes vs. intrinsic I-R tolerance.

Reversal of IPC effects on apoptotic mediators may also contribute to impaired cardioprotection in T2D. Whereas IPC reduced post-ischaemic Bax and Bak in hearts from healthy mice, Bax expression was increased by IPC in hearts from T2D mice. Previous studies demonstrating blunted protective responses with T2D report modest reductions or no change (Gao et al., 2016) in apoptosis, while a parallel reduction in apoptosis is reported when protection is preserved (Ghaboura et al., 2011; Peake et al., 2013). Supporting our observation that disease may not simply suppress but actually reverse the effects of myocardial conditioning stimuli on injury determinants, Przyklenk et al. detailed worsened myocardial injury with post-conditioning, although in T1D as opposed to T2D (Przyklenk et al., 2011).

Limitations

Two study limitations are worth noting. First, we quantitate protein expression after
ischemic insult, when injury and cell death manifest. Although data reveal no differences at this time that are consistent with I-R intolerance, exaggerated injury with T2D could involve differences pre-ischaemia (influencing propensity to subsequent cell injury/death). There is evidence of changes in baseline Bcl2 proteins in models of T2D, differences that may be maintained or lost with I-R (Cheng et al., 2018). Findings are equivocal regarding baseline phospho-AKT, including evidence of no change (Cheng et al., 2018; Desrois et al., 2004), reductions (Chen et al., 2016) or increases (Bouhidel et al., 2008; Cook et al., 2010) in T2D. Although post-ischaemic AKT phosphorylation was unaltered by diabetes (Figure 5), it is possible earlier inhibitory or stimulatory impacts of T2D may normalise by 45 min reperfusion. That said, studies indicate baseline differences are preserved or exaggerated in reperfused tissue (Bouhidel et al., 2008; Chen et al., 2016; Cheng et al., 2018; Liu et al., 2013).

Secondly, we have assessed outcomes in male animals, and despite healthy female myocardium exhibiting superior I-R tolerance (Reichelt et al., 2009), females appear specifically sensitised to the cardiovascular impacts of diabetes (Reichelt et al., 2013), including greater mortality after infarction (Roffi et al., 2013). Few experimental studies contrast male and female responses, however Desrois et al. report myocardial I-R tolerance is selectively impaired by diabetes in aging female but not male rats, an effect independent of phospho-AKT levels (Desrois et al., 2004). Influences of sex on the cardiac and coronary impacts of T1D and T2D warrant focussed study (Reichelt et al., 2013).

Conclusions

The current study reveals distinct myocardial outcomes in models of chronic T1D and T2D, with I-R tolerance and IPC generally preserved in the former vs. impaired in the latter. Indeed, normally protective IPC may augment injury markers and mechanisms (including
nitrosative stress and apoptotic signalling) in the context of T2D. Thus, while prolonged and significant hyperglycaemia in adult male mice does not impair myocardial I-R tolerance, lesser hyperglycaemia combined with insulin-resistance and neuroendocrine dysfunction in T2D is detrimental to the hearts ability to withstand injury and execute intrinsic protective responses.

Disclosures

Availability of data and material All data is available from the corresponding author upon reasonable request.

Competing interests No conflict of interest declared.

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Author Contributions:

JSR and JPH – conception, design, acquisition and interpretation of data, drafting and editing of the manuscript; JSR and JPH are the guarantors of this work.

TAG and TH - acquisition and analysis of data, and reviewed and edited the manuscript

EFDT and JNP - analysis and interpretation of data, review and editing of the manuscript
References


FIGURE CAPTIONS

**Figure 1**  Body weight and metabolic parameters in non-diabetic (CTRL), T1D and type T2D mice. Data are shown for: A) final body weight, B) % weight gain over the 12 week experimental protocol, C) fasting glucose, D) GTT AUC, E) fasting insulin, and F) HOMA-IR. Results presented as means±SD (T1D or T2D, n=18 each; CTRL, n=29). **, P<0.01; ***, P<0.001; ****, P<0.0001 vs. CTRL.
Figure 2  Endocrine factors in non-diabetic (CTRL), T1D and T2D mice. Data are shown for circulating: A) leptin, B) ghrelin, C) resistin and E) noradrenaline. Results presented as means±SD (T1D or T2D, n=6-7; CTRL, n=13). *, P<0.05; ***, P<0.001 vs. CTRL.
Figure 3  Ischaemic contracture in un-treated (-IPC) and preconditioned (+IPC) hearts from non-diabetic (CTRL), T1D and T2D mice. Data are shown for: A) peak contracture and B) time to reach peak contracture. Results presented as means±SD (T1D, non-IPC, n=10; T1D, IPC, n=8; T2D, non-IPC, n=10; T2D, IPC, n=8; CTRL, non-IPC, n=21; CTRL, IPC, n=8). *, P<0.05; **, P<0.01 vs. CTRL or -IPC.
Figure 4  Post-ischaemic outcomes in un-treated (-IPC) and preconditioned (+IPC) hearts from non-diabetic (CTRL), T1D and T2D mice. Data are shown for post-ischaemic: A) LVDP, B) EDP, C) final coronary flow per gram heart (% baseline), and D) TnI efflux (μg TnI/g heart). Results presented as means±SD (Functional outcomes: T1D, non-IPC, n=10; T1D, IPC, n=8; T2D, non-IPC, n=10; T2D, IPC, n=8; CTRL, non-IPC, n=21; CTRL, IPC, n=8. TnI efflux: n=6-8/group). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 vs. CTRL or -IPC.
Figure 5  Post-ischaemic Bax, Bak, AKT phosphorylation and cardiac nitrosylation in untreated (-IPC) and preconditioned (+IPC) hearts from non-diabetic (CTRL), T1D and type T2D mice. Data shown for: A) pro-apoptotic Bax, B) pro-apoptotic Bak, C) cardioprotective AKT phosphorylation, and D) cardiac nitrotyrosine levels. Results presented as means±SD (Protein expression:  \( n=5-8 \)/group. Nitrotyrosine: T1D, non-IPC,  \( n=9 \); T1D, IPC,  \( n=7 \); T2D, non-IPC,  \( n=8 \); T2D, IPC,  \( n=7 \); CTRL, non-IPC,  \( n=14 \); CTRL, IPC,  \( n=7 \)). *, P<0.05; ***, P<0.001 vs. CTRL or -IPC.
Figure 6  Relationships between I-R outcomes and A) ischaemic contracture or B) cardiac nitrotyrosine levels in non-diabetic, T1D and T2D hearts. Data are shown for functional (LVDP as % of baseline) and cell death (TnI efflux) outcomes. Linear least-squares regression was undertaken on mean data (curve fits shown). Data are means±SD.
Table 1  Summary of systemic and myocardial effects of chronic T1D and T2D in mice.

<table>
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<tr>
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<th>Type 1 Diabetes</th>
<th>Type 2 Diabetes</th>
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<td>Body Weight</td>
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<td><strong>MYOCARDIAL</strong></td>
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<td>Ischaemic Contracture</td>
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<td>I-R Contractile Dysfunction</td>
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<td>I-R Oncosis (TnI efflux)</td>
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<td>Ischaemic Contracture</td>
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<td>I-R contractile Dysfunction</td>
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↓, reduced; ⇔, no effect; ↑, increased. Additional arrows indicate greater effect.