Obesity improves myocardial ischaemic tolerance and RISK signalling in insulin-insensitive rats

Daniel Donner1, John P. Headrick1, Jason N. Peart1 and Eugene F. du Toit1,*

SUMMARY

Obesity with associated metabolic disturbances worsens ischaemic heart disease outcomes, and rodent studies confirm that obesity with insulin-resistance impairs myocardial resistance to ischemia-reperfusion (I-R) injury. However, the effects of obesity per se are unclear, with some evidence for paradoxical cardioprotection (particularly in older subjects). We tested the impact of dietary obesity on I-R tolerance and reperfusion injury salvage kinase (RISK) signalling in hearts from middle-aged (10 months old) insulin-insensitive rats. Hearts from Wistar rats on either a 32-week control (CD) or high carbohydrate obesogenic (OB) diet were assessed for I-R resistance in vivo (45 minutes left anterior descending artery occlusion and 120 minutes reperfusion) and ex vivo (25 minutes ischemia and 60 minutes reperfusion). Expression and δ-opioid receptor (δ-OR) phospho-regulation of pro-survival (Akt/PKB, Erk1/2, eNOS) and pro-injury (GSK3β) enzymes were also examined. OB rats were heavier (764±25 versus 657±22 g for CD; \(P<0.05\)), hyperleptinaemic (11.1±0.7 versus 5.0±0.7 for CD; \(P<0.01\)) and comparably insulin-insensitive (HOMA-IR of 63.2±3.3 versus 63.2±1.6 for CD). In vivo infarction was more than halved in OB (20±3%) versus CD rats (45±6%; \(P<0.05\)), as was post-ischaemic lactate dehydrogenase efflux (0.4±0.3 mU/ml versus 5.6±0.5 mU/ml; \(P<0.02\)) and ex vivo contractile dysfunction (62±2% versus 44±6% recovery of ventricular force; \(P<0.05\)). OB hearts exhibited up to 60% higher Akt expression, with increased phosphorylation of eNOS (+100%), GSK3β (+45%) and Erk1/2 (+15%). Pre-ischaemic δ-OR agonism with BW373U86 improved recoveries in CD hearts in association with phosphorylation of Akt (+40%), eNOS (+75%) and GSK3β (+30%), yet failed to further enhance RISK-NOS activation or I-R outcomes in OB hearts. In summary, dietary obesity in the context of age-related insulin-insensitivity paradoxically improves myocardial I-R tolerance, in association with moderate hyperleptinaemic and enhanced RISK expression and phoso-regulation. However, OB hearts are resistant to further RISK modulation and cardioprotection via acute δ-OR agonism.

INTRODUCTION

Obesity might double the risk of coronary artery disease and heart failure (Kenchaiah et al., 2002) and worsens outcomes from myocardial infarction (aronson et al., 2010; Clavijo et al., 2006). These negative influences are consistent with experimental evidence of ischaemic intolerance and repressed cardioprotection in hearts from obese, dyslipidaemia and insulin-resistant animals (Bouhid et al., 2008; Clark et al., 2011; du Toit et al., 2005; du Toit et al., 2008; Essop et al., 2009; Katakam et al., 2007; Maarman et al., 2012; Morel et al., 2003). However, obesity is complex, associated with co-morbidities including dyslipidaemia, inflammation and oxidative stress, insulin-insensitivity or overt diabetes with hyperglycaemia. Contributions of such factors to the overall effects of obesity remain unclear. We have reported that adiposity is not a reliable predictor of ischaemia-reperfusion (I-R) tolerance (du Toit et al., 2008), and different potential determinants might be modified independently of obesity (Aubin et al., 2008; Aubin et al., 2010). Pro-inflammatory cytokine signalling could link adiposity to cardio-metabolic complications (Savage et al., 2005) and to I-R intolerance, with increased tumour necrosis factor α (TNF-α), for example, inhibiting insulin receptor substrate 1 (IRS-1) and both insulin (hirosumi et al., 2002) and protective Akt signalling (chuang et al., 2011). Dyslipidaemia could be important, though data are mixed: hypercholesterolemia with cholesterol-rich diets can improve I-R tolerance (while impairing post-conditioning) (girod et al., 1999; Lauzier et al., 2009), exaggerate myocardial infarction (szucs et al., 2011) or exert no effect (görbe et al., 2011). Hyperglycaemia, arising from insulin insensitivity, can either promote myocardial cell death (cai et al., 2002; Fiordaliso et al., 2001; Shizukuda et al., 2002) or inhibit myocardial I-R injury (champattanachai et al., 2007; Schaffer et al., 2000; Xu et al., 2004). Insulin-sensitivity, on the other hand, consistently impacts I-R tolerance and might well be the primary determinant of negative effects of complex obesity. Resistance to I-R injury is impaired in non-obese models of insulin-resistance (Morel et al., 2003), in dietary obesity with insulin-resistance (du Toit et al., 2005; du Toit et al., 2008; Maarman et al., 2012) and in obese Zucker rats with insulin-resistance (Katakam et al., 2007) or diabetes (yu et al., 2005). Desrosis and colleagues also present evidence linking insulin-resistance to I-R intolerance in diabetic Goto-Kakizaki (GK) rats (Desrosis et al., 2010), and we report association between insulin-resistance and cardiac injury in male but not in female rats (Clark et al., 2011). Thus, insulin-resistance consistently impairs cardiac I-R tolerance, which probably underpins the negative cardiac effects of obesity and metabolic syndrome. In contrast, the effects of obesity per se remain unclear.

Though generally considered detrimental (potentially due to associated insulin-resistance and other abnormalities), there is also evidence of cardiovascular benefit with obesity in humans (an ‘obesity paradox’), i.e. reduced morbidity and mortality in overweight or obese patients with ischaemic heart disease or heart...
Obesity and cardiac ischaemic tolerance

**Clinical issue**

Human obesity is a complex disorder usually associated with many other metabolic abnormalities. Most of these co-morbidities (e.g. dyslipidaemia, insulin resistance, hyperglycaemia, oxidative stress and inflammation) can influence myocardial sensitivity to injury or disease. However, the effects of obesity on the heart have been unclear; for example, some studies reported that obesity confers a cardioprotective effect to ischemia-reperfusion injury. To optimally manage cardiovascular outcomes in obese individuals, it is thus important to identify the effects on the heart of individual obesity-associated factors.

**Results**

In this study, the authors use an insulin-insensitive rat model of dietary obesity to investigate whether obesity per se is a key factor compromising myocardial tolerance to ischemia. Their findings indicate that obesity with mild hyperleptinaemia does not compromise myocardial tolerance to ischemia; rather, obesity might even be beneficial, at least in this setting of insuling resistance. Additional data suggest that cardioprotection conferred by obesity might occur via modulation of reperfusion injury salvage kinase (RISK) signalling. Based on these data and their previous findings, the authors propose that insulin resistance is the primary factor that impairs resistance to myocardial ischemia.

**Implications and future directions**

These data show that obesity per se does not compromise myocardial tolerance to ischemia in insulin-insensitive rats with dietary obesity. These data provide clues about highly debated influence of human obesity on cardiovascular health and disease. The delineation of underlying mechanisms in future studies should help to develop therapies that limit the detrimental effects of obesity-associated insulin resistance on the heart.

**TRANSLATIONAL IMPACT**

**Clinical issue**

Obesity-dependent protection remains controversial. Nonetheless, the relevance and indeed existence of these protective effects appear age-related. Hyperleptinaemia does not compromise myocardial tolerance to ischemia, but, rather, obesity might even be beneficial, at least in this setting of insulin resistance. Additional data suggest that cardioprotection conferred by obesity might occur via modulation of reperfusion injury salvage kinase (RISK) signalling. Based on these data and their previous findings, the authors propose that insulin resistance is the primary factor that impairs resistance to myocardial ischemia.

**Implications and future directions**

These data show that obesity per se does not compromise myocardial tolerance to ischemia in insulin-insensitive rats with dietary obesity. These data provide clues about highly debated influence of human obesity on cardiovascular health and disease. The delineation of underlying mechanisms in future studies should help to develop therapies that limit the detrimental effects of obesity-associated insulin resistance on the heart.

**RESULTS**

**Baseline characteristics of obese and lean animals**

Wistar rats were kept for 32 weeks on control (CD) or high carbohydrate obesogenic (OB) diets. Rats on the OB diet were 16% heavier, with larger visceral fat reserves than CD litter-mates (Table 1). OB rats also exhibited elevated triglycerides, with comparable insulin and glucose compared with lean CD rats. Consistent with prior studies, 10-month-old Wistar rats displayed elevated serum insulin and homeostatic model assessment of insulin resistance (HOMA-IR) levels compared with 3-month-old rats (Gómez-Pérez et al., 2011; Gu et al., 2011). Serum insulin levels in young rats were 50% of those in 10-month-old CD and OB rats (3.04±0.10 versus 6.70±0.18 and 6.86±0.11 ng/ml in young versus old CD and OB rats, respectively). Furthermore, OB rats exhibited normal serum TNF-α and insulin-like growth factor 1 (IGF-1) levels, with significantly elevated leptin levels (Table 1). In vivo arterial pressures and heart rates were comparable between CD and OB groups (Table 2).

**Table 1. Biometric data for rats fed on control (CD) and high carbohydrate (OB) diets**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD rats</th>
<th>OB rats</th>
<th>n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>659.2±20.42</td>
<td>776±21.45</td>
<td>32</td>
<td>0.0046</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.37±0.04</td>
<td>1.52±0.03</td>
<td>14</td>
<td>0.0043</td>
</tr>
<tr>
<td>Visceral adiposity (% body weight)</td>
<td>5.48±0.28</td>
<td>7.72±0.35</td>
<td>32</td>
<td>2.0E-05</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.27±0.12</td>
<td>1.69±0.14</td>
<td>10</td>
<td>0.0321</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>0.68±0.07</td>
<td>0.66±0.08</td>
<td>9</td>
<td>0.8459</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.86±0.31</td>
<td>9.1±0.4</td>
<td>10</td>
<td>0.6282</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>6.70±0.18</td>
<td>6.9±0.1</td>
<td>6</td>
<td>0.4836</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>63.2±3.3</td>
<td>63.2±3.3</td>
<td>6</td>
<td>0.9837</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>4960±747</td>
<td>11,106±671</td>
<td>7</td>
<td>2.72E-05</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>33.1±8.4</td>
<td>24.5±4.1</td>
<td>7</td>
<td>0.3787</td>
</tr>
<tr>
<td>IGF-1 (pg/ml)</td>
<td>6601±417</td>
<td>6535±244</td>
<td>10</td>
<td>0.8949</td>
</tr>
</tbody>
</table>

Data were measured in CD and OB rats at sacrifice. Values are means ± s.e.m.
Normoxic cardiac performance and coronary perfusion assessed ex vivo did not differ between CD and OB hearts (Fig. 1; Table 2). Left ventricular systolic, diastolic and developed pressures were similar across groups, as was the positive differential of force development (+dP/dt) and coronary flow rate. Absence of changes in baseline function ex vivo was consistent with unaltered arterial diastolic, systolic and mean pressures in vivo (Table 2).

Obesity improves cardiac I-R tolerance while eliminating δ-OR cardioprotection

Global I-R significantly depressed contractile function in vitro (Fig. 1), with reduced ventricular pressure development and a sustained increase in diastolic pressure. Post-ischaemic contractile recovery was incomplete in both CD and OB hearts, but significantly better in OB than in CD hearts (Fig. 1A). Recovery of inotropic state, as assessed by +dP/dt, was also significantly improved in hearts from OB rats, whereas post-ischaemic efflux of cardiac lactate dehydrogenase (LDH) was significantly reduced (Fig. 1B,C). Pre-ischaemic δ-OR agonism with BW373U86 had no effect on post-ischaemic mechanical function in either CD or OB hearts (Fig. 1A). However, δ-OR agonism significantly reduced LDH efflux in CD but not OB hearts (Fig. 1C).

Analysis of myocardial infarction in vivo revealed that ~45% of the area at risk (AAR) was infarcted in hearts from CD animals, with infarction in OB hearts more than halved to ~20% (Fig. 2). δ-OR agonism also decreased infarction by ~40% in CD hearts, yet failed to modify infarct size in OB hearts (Fig. 2). The AAR was similar across all groups (CD, 34.7±5.1%; CD+BW373U86, 31.0±5.3%; OB, 20.7±2.1%).

Table 2. Measures of basal mechanical function in in vivo and ex vivo hearts from rats fed on control (CD) and high carbohydrate (OB) diets

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD rats</th>
<th>OB rats</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td><strong>In vivo</strong> (n=11-13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats/minute)</td>
<td>376±7</td>
<td>356±17</td>
<td>0.5831</td>
</tr>
<tr>
<td>Diastolic pressure (mm Hg)</td>
<td>109±7</td>
<td>106±9</td>
<td>0.9509</td>
</tr>
<tr>
<td>Systolic pressure (mm Hg)</td>
<td>140±9</td>
<td>137±10</td>
<td>0.9107</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>119±7</td>
<td>116±9</td>
<td>0.9982</td>
</tr>
<tr>
<td>MAP x heart rate (mm Hg/minute)</td>
<td>45277±3304</td>
<td>42460±4053</td>
<td>0.8538</td>
</tr>
<tr>
<td><strong>Ex vivo</strong> (n=8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary flow rate (ml/minute)</td>
<td>19.4±0.6</td>
<td>20.7±0.7</td>
<td>0.2087</td>
</tr>
<tr>
<td>Heart rate (beats/minute)</td>
<td>309±4</td>
<td>310±2</td>
<td>0.8132</td>
</tr>
<tr>
<td>LVDevP (mm Hg)</td>
<td>112±4</td>
<td>116±3</td>
<td>0.3568</td>
</tr>
<tr>
<td>dP/dt max (mm Hg/s)</td>
<td>4234±214</td>
<td>4627±161</td>
<td>0.1593</td>
</tr>
</tbody>
</table>

In vivo functional measures were made immediately prior to regional ischemia in anaesthetised CD and OB rats. Measures from ex vivo hearts were made under normoxic conditions, also immediately prior to global ischaemia. MAP, mean arterial pressure; LVDevP, left ventricular developed pressure; dP/dt max, peak positive differential of force development. Values are means ± s.e.m.

Fig. 1. Pre- and post-ischaemic contractile function and post-ischaemic LDH efflux in Langendorff perfused hearts from CD, OB, CD+BW373U86 and OB+BW373U86 rats. Hearts were untreated or pre-treated for 10 minutes pre-ischaemia with 1 μM BW373U86. (A) Left ventricular developed pressure (LVDevP) (**P<0.05 versus CD); (B) Post-ischaemic recovery of +dP/dt after 25 minutes of ischaemia and 60 minutes reperfusion (**P<0.05 versus CD); and (C) post-ischaemic efflux of myocardial LDH in the first and second minutes of reperfusion (**P<0.05,**P<0.02). Data are means ± s.e.m. (n=4).
These data collectively reveal that dietary obesity induces cardioprotection in older insulin-insensitive animals, an effect evident in vivo and ex vivo.

**Obesity improves RISK-NOS signalling in hearts from insulin-insensitive rats**

Dietary obesity induced significant changes in cardiac signalling proteins. The OB diet increased expression of Akt in both cytosolic and membrane compartments by 40-60%, without modifying total expression levels for other proteins (Fig. 3). Obesity had no effect on the phosphorylation state of Akt (Fig. 4). Furthermore, phosphorylation states of cytosolic glycogen synthase kinase 3β (GSK3β), membrane endothelial nitric oxide synthase (eNOS; only detected in the membrane fraction in all groups), and cytosolic extracellular signal-regulated kinases 1 and 2 (Erk1/2) were increased in OB hearts by 45, 100 and 15%, respectively (Figs 5, 6).

**Obesity attenuates δ-OR agonist activation of RISK-NOS signalling**

In terms of signalling responsiveness to δ-OR agonism in CD hearts, 1 μM BW373U86 treatment increased phosphorylation of cytosolic (but not membrane) Akt by 40% (Fig. 4); membrane (but not cytosolic) GSK3β by ~40% (Fig. 5); and membrane eNOS by 75% (Fig. 6). Cytosolic Erk1/2 was insensitive to activation (Fig. 6). Obesity eliminated signalling sensitivity to δ-OR agonism. In contrast to CD hearts, δ-OR agonism failed to increase phosphorylation of Akt, GSK3β or eNOS in OB hearts. Indeed, there was a small 15% decline in apparent phosphorylation of cytosolic Erk1/2 in the OB group (Fig. 6).

**DISCUSSION**

We have previously reported that obesity with features of metabolic syndrome (obesity, insulin-resistance, dyslipidaemia) decreases myocardial resistance to I-R compared with lean insulin-sensitive animals. Decreased ischaemic tolerance is reflected in worsened infarction (du Toit et al., 2005; du Toit et al., 2008; Essop et al., 2009; Maarman et al., 2012) and mechanical dysfunction (du Toit et al., 2005; du Toit et al., 2008). By contrast, the present study reveals that high-carbohydrate-induced obesity in older insulin-insensitive animals paradoxically improves myocardial I-R tolerance (compared with lean insulin-insensitive animals), an effect apparent in vivo and ex vivo. Improved I-R tolerance is associated with normal pro-inflammatory cytokine levels and similar glucose levels and/or insulin-insensitivity, moderate hyperleptinaemic, and improved expression and phospho-regulation of myocardial enzymes impacting on cell survival. However, δ-OR-triggered RISK activation and associated cardioprotection are eliminated by obesity in these animals. The data thus collectively indicate that obesity itself improves I-R tolerance in insulin-insensitive older rats, potentially via improved RISK-NOS signalling. However, enhanced

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Fig. 2. Myocardial infarct size following 45 minutes of LAD coronary artery occlusion and 120 minute reperfusion in situ in hearts of CD and OB, CD+BW373U86 and OB+BW373U86 rats. Animals received either vehicle or 1 mg/kg BW373U86 administered via the right jugular vein for 10 minutes prior to LAD occlusion. Data are means ± s.e.m. (n=6-7); *P<0.05, **P<0.02.

Fig. 3. Effects of dietary obesity on total myocardial expression of Akt, GSK3β, Erk1/2 and eNOS. Expression data are normalised to values for control (CD) hearts. Data are means ± s.e.m. (n=5); *P<0.05, **P<0.005.

Fig. 4. Effects of dietary obesity and BW373U86 pre-treatment on ratio of myocardial phosphorylated Akt to total Akt. Animals were treated with vehicle or 1 mg/kg BW373U86 administered via the right jugular vein for 10 minutes prior removal of the heart. (A) Cytosolic Akt ratios. (B) Membrane Akt ratios. For comparison, phosphorylation ratios are normalised to values from vehicle-treated control (CD) hearts. Data are means ± s.e.m. (n=5); *P<0.05.
basal expression and phospho-activation of RISK-NOS paths might also limit capacity to further protect via stimuli engaging the same signalling (e.g. δ-OR agonism).

Preserved contractile function with dietary obesity

The present data indicate that high-carbohydrate-driven dietary obesity does not negatively influence myocardial contractile function in insulin-sensitive rats (Fig. 1; Table 2). Data regarding contractile effects of obesity are scarce and equivocal, with evidence for and against obesity-dependent dysfunction. Obesity is an established independent risk factor for heart failure (Kenchaiah et al., 2002), though whether obesity directly promotes pump dysfunction is unknown. Some studies support impairment of systolic function (Peterson et al., 2004; Wong et al., 2004), whereas other data support preserved function in obese humans (Stoddard et al., 1992; Zarich et al., 1991), or negative effects due to shifts in cardiac loading (Alpert et al., 1993). Wilson et al. report mechanical dysfunction with an obesogenic Western diet of high fat and reduced carbohydrates (Wilson et al., 2007), whereas our prior work indicates that contractile function is compromised in obese insulin-resistant rats but not in lean insulin-sensitive rats (du Toit et al., 2005; du Toit et al., 2008). Ren et al. recently found that 16 weeks of a high-fat diet reduced isolated myocyte contractility, though presented no data regarding the myocardium (Ren et al., 2010). They also reported impaired contractility in the murine ob/ob model, in association with reduced phospho-activation of Akt and eNOS. Our data for dietary rather than genetically determined obesity, with a high carbohydrate rather than high fat diet, reveals a different outcome, with no shift in contractile function in intact beating hearts (Fig. 1; Table 2). Common arterial pressures in OB and CD rats also support preservation of contractile function in vivo (Table 2). It is possible that negative contractile effects of obesogenic diets arise from repression of Akt and/or NOS signalling (Ren et al., 2010), whereas baseline Akt expression and NOS phospho-activation are enhanced in the current model.

Dietary obesity enhances I-R tolerance in hearts from insulin-insensitive animals

Some clinical studies identify worsened outcomes from myocardial infarction with obesity (Kragelund et al., 2005; Rana et al., 2004; Sharma et al., 2009). Nonetheless, reduced morbidity and mortality and emergence of the ‘obesity paradox’ in overweight and obese patients (Hall et al., 2005; Lavie et al., 2009; Pingitore et al., 2007) indicate that there are situations where obesity can in fact prove protective. This might reflect differing roles of associated metabolic disturbances in determining cardiac outcomes. Myocardial I-R tolerance in insulin-insensitive rats was enhanced by obesity in vivo and ex vivo, supporting conservation of a protected phenotype from in situ to ex vivo blood-free hearts with fixed substrate supply. These data contrast prior studies in hearts from obese insulin-resistant rats exhibiting exaggerated post-ischaemic dysfunction (du Toit et al., 2005; du Toit et al., 2008) and infarction (Clark et al., 2011; du Toit et al., 2005; du Toit et al., 2008; Essop et al., 2009; Maarman et al., 2012). A key difference is that these studies compare obese insulin-resistant with lean insulin-sensitive rats, thus assessing combined effects of obesity and insulin-resistance. Multiple studies confirm that insulin-resistance, isolated (Morel et al., 2003) or obesity-related (Clark et al., 2011; du Toit et al., 2005; du Toit et al., 2008; Essop et al., 2009; Maarman et al., 2012).
show that dietary obesity with mild hyperleptinaemic significantly improves expression and activation of Akt, phospho-activation of eNOS and Erk1/2, and phospho-inhibition of GSK3β (Figs 4–6) in myocardium from insulin-insensitive animals. Observed changes are collectively consistent with involvement in obesity-dependent protection against I-R injury.

Expression of Akt was increased in both cytosol and membrane fractions (Fig. 3). Because Akt activation involves cytosol to membrane translocation, increased expression in both compartments reflects an increase in both intrinsically active Akt and the capacity for further activation of Akt signalling. eNOS expression was largely restricted to the membrane, where phosphorylation was increased in response to obesity and BW373U86 (Fig. 6). This is consistent with known caveolar localisation of cardiac eNOS, essential to its protective functions (caveolae and caveolins regulating protective eNOS activation against injurious uncoupling for example). By contrast, expression of phosphorylated (inactive) GSK3β was increased in the cytosol but not in the membrane fraction (Fig. 5), consistent with concentration of de-phosphorylated (active) GSK3β in nuclear and mitochondrial compartments, where it promotes cell death. Based on their known actions, upregulation of Akt and activation of eNOS and Erk1/2 is predicted to limit myocardial stunning, oxidative stress, mitochondrial dysfunction and apoptotic and oncotic pathways of cell death. Similarly, enhanced inhibition of GSK3β is predicted to limit mitochondrial dysfunction and cell death (Juhaszova et al., 2009). Thus, observed changes in expression and phosphorylation of these molecules are likely to contribute to the obesity-dependent protection against I-R injury reported here.

How might beneficial adaptations in Akt, Erk1/2, NOS and GSK3β signalling be achieved? Improved phospho-regulation could stem from markedly increased expression (and activation) of upstream Akt, which influences multiple pathways (Bertrand et al., 2008; Chang et al., 2010) and is sensitive to and regulates substrate metabolism (Pagano et al., 1981; Smith and Yellon, 2011). Enhanced phosphorylation of Erk1/2, NOS and GSK3β could thus all stem from increased upstream expression of active Akt. As noted above, elevated leptin (Table 1) might also protect via activation of phosphoinositide 3-kinase (PI3K)-Akt and Erk1/2 (Smith and Yellon, 2011; Smith et al., 2006). Although leptin protection might be limited in extreme models of obesity (Smith and Yellon, 2011), benefit could be preserved in obesity with insulin-sensitivity, and absence of elevations in TNF-α could further promote Akt-dependent protection. However, although leptin can contribute to Akt and Erk1/2 phosphorylation, its elevation is an unlikely explanation for increased Akt expression (Ren et al., 2010) or GSK3β phosphorylation in cardiac cells (Palanivel et al., 2006). The basis of these latter effects awaits further investigation.

**Dietary obesity renders hearts refractory to δ-OR activation of signalling and protection**

In addition to assessing baseline changes in RISK-NOS signalling, we contrasted the responsiveness of RISK-NOS signalling to a conventional protective stimulus engaging these paths, i.e. δ-OR agonism (Fryer et al., 2001; Gross et al., 2007a). Several studies indicate that hearts from obese insulin-resistant (Katakam et al., 2007) or diabetic (Bouhidel et al., 2008) animals are refractory to protective interventions targeting RISK signalling and potentially involving δ-
ORs. Wagner and co-workers also report that obese rats with metabolic syndrome are refractory to post-conditioning (also potentially involving ORs) (Wagner et al., 2008). Such studies (Bouhidel et al., 2008; Katakam et al., 2007; Wagner et al., 2008) suggest that altered RISK functionality might contribute to impaired efficacy of conventional protective strategies. We confirm here that hearts from obese insulin-insensitive animals are unresponsive to δ-OR agonism – neither RISK activation nor protection observed in CD hearts was evident in OB tissue. The basis of this insensitivity remains to be more directly assessed, though interestingly we have also shown that OR engagement of myocardial protein kinase signalling is inhibited with aging, in association with impaired cardioprotection (Pearl et al., 2007). Age and dietary obesity might thus exert similar inhibitory effects on δ-OR signal transduction. In addition, substantial upregulation and activation of baseline RISK-NOS signalling in OB hearts (e.g. more than a 100% increase in eNOS phospho-activation and 50% increase in GSK3β phospho-inhibition) might render these pathways relatively refractory to further activation. The level of protection with dietary activation of RISK-NOS paths could also be near maximal, consistent with the profound 55% reduction in infarct size in OB hearts, comparable to or exceeding protection with δ-OR agonism in CD hearts (Fig. 2), and the protection typically observed with pre- or post-conditioning. Thus, although obesity might induce some benefit, these very effects might also render hearts resistant to further protective intervention.

Limitations
The primary limitation of the current study is that although we assessed cardiac injury and infarction in vivo and in vitro (observing parallel responses), we did not assess longer term outcomes beyond 2 hours of reperfusion. It is thus feasible that differences detected at 2 hours reflect delayed rather than overall reduced cardiac injury. However, this is highly unlikely given that evidence of infarct development in this model is essentially complete within 1-2 hours of reperfusion (Marais et al., 2005; Schwarz et al., 2000), limiting the potential for subsequent emergence of differences in cell death. A second limitation is that we were unable to identify the specific basis of shifts in RISK-NOS signalling in the current work. Although a modest twofold elevation in leptin could contribute to activation of Akt and other signal components, this cannot explain markedly increased Akt expression, nor is unaltered Erk1/2 activation consistent with leptin involvement. Future work should more directly address the molecular mechanisms contributing to improvements in RISK-NOS signalling with obesity.

Conclusions
Dietary obesity (with hyperleptinaemic) protects older insulin-insensitive hearts against I-R injury when compared with hearts from lean insulin-insensitive animals, an effect that could contribute to a so-called ‘obesity paradox’ in humans (Arena and Lavie, 2010; Barbarroja et al., 2010; Dorner and Rieder, 2012; Hall et al., 2005; Lavie et al., 2009; Pingitore et al., 2007). Cardioprotection with obesity in this setting is associated with improved expression and phospho-activation of the PI3K-Akt-eNOS pathway, together with phospho-inhibition of injurious GSK3β. Although these changes might contribute to obesity-dependent protection, they might also render hearts refractory to further protection via receptor stimuli (e.g. δ-OR agonism) targeting the same PI3K-Akt-eNOS signalling. These findings thus support initial beneficial adaptations in I-R tolerance and pro-survival signalling with obesity per se, and implicate transition to insulin-resistance and ultimately diabetes as the key determinant of the negative influences of complex obesity (or metabolic syndrome) on myocardial stress resistance.

MATERIALS AND METHODS

Animals and experimental design
All studies were performed in accordance with the guidelines of the Animal Ethics Committee of Griffith University, which is accredited by the Queensland Government, Department of Primary Industries and Fisheries under the guidelines of The Animal Care and Protection Act 2001, Section 757.

Sixty-four 8-week-old male Wistar rats (~200 g) were placed on either a control (CD) or high carbohydrate obesogenic (OB) diet for 32 weeks. Fresh food was provided daily, with rats having ad libitum access to water and standard rat chow (carbohydrate 73%; protein 18%; fat 9%) for the CD group, or rat chow supplemented with sweetened condensed milk (Nestle, Australia) and raw sugar (carbohydrate 82%; protein 11%; fat 7%) for the OB group. Rats were supplied by the Animal Resources Centre (Canning Vale, Australia), and housed at 23°C with a 12-hour day-night cycle in an on-site animal facility.

Rats were randomly divided into either CD or OB groups (n=32). OB rats become viscerally obese whereas CD rats remained lean (Table 1). Both groups of middle-aged animals were insulin-insensitive (Table 1), consistent with recent observations in the Wistar rat (Gómez-Pérez et al., 2011; Gu et al., 2011). Within each experimental group, 14 rats were randomly selected for assessment of infarct development in vivo and 8 rats for ex vivo assessment of functional responses to I-R. The remaining 10 rats per group were used for biochemical and western blot analysis. Of the 14 rats from each in vivo study group, 7 were pre-treated with vehicle and 7 were pre-treated with a δ-OR agonist (BW373U86). Of the 8 rats from each ex vivo study group, 4 were pre-treated with vehicle and 4 were pre-treated with a δ-OR agonist (BW373U86).

Ex vivo perfused hearts
Rats were anaesthetised (60 mg/kg sodium pentobarbital i.p.) and hearts perfused in a Langendorff mode as described in detail previously (du Toit et al., 2005; du Toit et al., 2008; Eissop et al., 2009; Maarman et al., 2012). Briefly, hearts were excised into ice-cold perfusion fluid, the aorta cannulated and coronary vessels perfused at a pressure of 100 cm H2O with modified Krebs-Henseleit buffer containing: 118 mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, 1.75 mM CaCl2, 1.2 mM MgSO4, 11 mM D-glucose and 0.5 mM EDTA. Perfusion fluid was saturated with 95% O2 and 5% CO2 at 37°C, giving a pH of 7.4. A fluid-filled polyvinyl chloride film balloon was inserted into the left ventricle and connected to a P23XL pressure transducer (Viggo-Spectramed, Oxnard, CA) for measurement of ventricular pressure. Balloon volume was adjusted to an initial end-diastolic pressure of 4-6 mm Hg and then left unchanged. Functional data were recorded at 1 KHz on a four-channel MacLab system (ADInstruments, Castle Hill, Australia) connected to an Apple iMac computer. Peak systolic, diastolic and developed pressures, heart rate, and rates of contraction or relaxation over time (dP/dt) were documented. Coronary flow was monitored via volumetric analysis of coronary effluent.
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After balloon placement, hearts were immersed in a water-jacketed chamber maintained at 37°C. Temperature of perfusion fluid was monitored by a needle thermometer probe (Model 52II, Fluke Corporation, Everett, Western Australia) in the coronary sinus. Hearts were excluded from study after a 15-minute stabilisation period if they failed to meet any of the following functional criteria: (i) coronary flow >25 ml/minute, (ii) unstable (fluctuating) contractile function, (iii) left ventricular systolic pressure <90 mmHg or (iv) significant cardiac arrhythmias. After an initial 15-minute period of normoxic stabilisation, hearts were switched to ventricular pacing at 300 beats/minute (Grass S9 stimulator, Quincy, MA), normalising rate to allow comparison of rate-dependent measures of contractile function (systolic and developed ventricular pressures) and inotropic state (dP/dt). Baseline measures were made after an additional 10-minute stabilisation, before subjecting hearts to 25 minutes of normothermic global zero-flow ischemia followed by 60 minutes of aerobic reperfusion. PACing was terminated on initiation of ischemia and resumed after 5 minutes of reperfusion. As a measure of myocardial injury or oncosis, coronary effluent was collected throughout reperfusion for analysis of total cardiac LDH efflux. Hearts that were untreated (0.9% saline vehicle) or pre-treated for 10 minutes of pre-ischemia with 1 μM of δ-OR agonist (BW373U86; Tocris Bioscience, Bristol, UK) in order to activate RISK signalling and associated protection (Fryer et al., 2001; Gross et al., 2007a; Peart et al., 2007).

In vivo myocardial infarction
Rats were anaesthetised (sodium pentobarbital, 60 mg/kg i.p.), intubated and ventilated (Harvard Instruments, Model 683), and placed on an adjustable heating pad to maintain a core temperature of 36-37°C, as monitored by rectal thermometer (Model 52II, Fluke Corporation, Everett, Western Australia). Maintenance of anaesthesia was monitored by assessing pedal withdrawal reflexes at 10-minute intervals. The carotid artery was cannulated for measurement of heart rate and arterial diastolic, systolic and mean pressures. A thoracotomy and reversible left anterior descending (LAD) artery ligation were performed as outlined previously (Clark et al., 2011). Animals received either 0.9% saline (vehicle) or 1 mg/kg BW373U86 administered via the right jugular vein for 10 minutes prior to removal of the heart. Hearts were removed and immediately snap-frozen in liquid nitrogen and stored at ~80°C.

Western immunoblot analyses
Left ventricular tissue samples were homogenised in lysis buffer (50 mmol/l HEPES, 150 mmol/l NaCl, 1.5 mmol/l MgCl2, 1 mmol/l EGTA, 10% glycerol plus 8.6 μmol/l leupeptin, 5.8 μmol/l pepstatin A, 4 mmol/l phenylmethylsulfonyl fluoride, 0.6 μmol/l aprotinin, 4 mmol/l sodium fluoride, and 0.8 mmol/l sodium orthovanadate) and centrifuged at 10,000 g for 15 minutes to remove nuclei and debris. Because protein activation processes might involve translocation from soluble to particulate fractions, we investigated expression of total and phosphorylated protein in both membrane and cytosolic fractions. The supernatant was centrifuged at 10,000 g to enrich for the cytosolic fraction, with the pelletted membrane fraction re-suspended by ultrasonication in a lysis buffer containing 1% Triton X-100. Fraction protein concentrations were determined by the Pierce assay. Equal quantities of protein (35 μg) were subsequently loaded onto 10% Tris-HCl gels and after electrophoresis (150 V, 1.5 hours) were transferred to PVDF membranes (50 V, 2 hours). Membranes were blocked with 3% BSA solution followed by probing overnight with antibodies (Cell Signaling Technology, Danvers, MA) for total or phosphorylated Akt (Ser473), Erk1/2 (p42/p44), GSK-3β (Ser9) and eNOS (Ser1177) followed by secondary antibody application (1:2500, Cell Signaling; 1:10,000, Santa Cruz Biotechnology) and enhanced chemiluminescence. Equal sample loading was confirmed by co-analysis of actin or GAPDH. Protein was detected by X-ray film, and densitometry assessed using NIH Image 1.63, with total expression and phosphorylation ratios normalised to values for untreated control (CD) tissue for the purposes of comparison across groups.

Biochemical analyses
Animals set aside for biochemical analyses were fasted for 10 hours before anaesthesia, blood collection and heart excision. Peritoneal and retro-peritoneal fat was removed and weighed. Serum triglycerides, high density lipoprotein (HDL) cholesterol and glucose levels were determined in fresh whole-blood using a Blood-Lipid and Glucose Analyser (L.D.X. Cholesterol Analyser, Alere, Australia). For serum analysis, remaining blood samples were placed in serum separation tubes (BD Vacutainer tubes), centrifuged and the serum stored at −80°C for later analysis. Levels of insulin (ALPCO Immunoassays, Salem, NH), leptin (Life Research, Australia), TNF-α (Life Research, Australia) and IGF-1 (Igf-1; life research, Australia) were determined in 96-well enzyme-linked immunosorbant assays (ELISAs) according to manufacturer instructions. Coronary effluent LDH activity was determined spectrophotometrically, as described previously (Peart and Headrick, 2003). To assess insulin resistance in lean and obese animals, the HOMA index was measured. Fasting blood glucose and insulin levels were used to determine the HOMA index using the standard formula: [fasting insulin (μIU/ml) × fasting glucose (mmol/l)] / 22.5.

For myocardial tissue sample collection, animals were treated with 0.9% saline (vehicle) or 1 mg/kg BW373U86 administered via the right jugular vein for 10 minutes prior to removal of the heart. Hearts were removed and immediately snap-frozen in liquid nitrogen and stored at ~80°C.

Statistical analyses
Unless otherwise stated, all results are expressed as means ± s.e.m. A two-tailed Student’s t-test was employed to contrast measures between CD and OB groups. Multi-way ANOVA with Tukey’s HSD test was used for comparison of δ-OR responses in CD and OB hearts. Evidence of statistical significance was accepted for P<0.05.


Obesity and cardiac ischaemic tolerance


