Cardiac mTOR rescues the detrimental effects of diet-induced obesity in the heart after ischemia-reperfusion

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Aoyagi T, Higa JK, Aoyagi H, Yorichika N, Shimada BK, Matsui T. Cardiac mTOR rescues the detrimental effects of diet-induced obesity in the heart after ischemia-reperfusion. Am J Physiol Heart Circ Physiol 308: H1530–H1539, 2015. First published April 17, 2015; doi:10.1152/ajpheart.00008.2015.—Diet-induced obesity deteriorates the recovery of cardiac function after ischemia-reperfusion (I/R) injury. While mechanistic target of rapamycin (mTOR) is a key mediator of energy metabolism, the effects of cardiac mTOR in ischemic injury under metabolic syndrome remains undefined. Using cardiac-specific transgenic mice overexpressing mTOR (mTOR-Tg mice), we studied the effect of mTOR on cardiac function in both ex vivo and in vivo models of I/R injury in high-fat diet (HFD)-induced obese mice. mTOR-Tg and wild-type (WT) mice were fed a HFD (60% fat by calories) for 12 wk. Glucose intolerance and insulin resistance induced by the HFD were comparable between WT HFD-fed and mTOR-Tg HFD-fed mice. Functional recovery after I/R in the ex vivo Langendorff perfusion model was significantly lower in HFD-fed mice than normal chow diet-fed mice. mTOR-Tg mice demonstrated better cardiac function recovery and had less of the necrotic markers creatine kinase and lactate dehydrogenase in both feeding conditions. Additionally, mTOR overexpression suppressed expression of proinflammatory cytokines, including IL-6 and TNF-α, in both feeding conditions after I/R injury. In vivo I/R models showed that at 1 wk after I/R, HFD-fed mice exhibited worse cardiac function and larger myocardial scarring along myofibers compared with normal chow diet-fed mice. In both feeding conditions, mTOR overexpression preserved cardiac function and prevented myocardial scarring. These findings suggest that cardiac mTOR overexpression is sufficient to prevent the detrimental effects of diet-induced obesity on the heart after I/R, by reducing cardiac dysfunction and myocardial scarring.

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Obesity is a major factor in metabolic syndrome, which increases the risk of type 2 diabetes mellitus and cardiovascular disease (2, 17). Metabolic syndrome is described as the presence of multiple metabolic risk factors, including obesity, dyslipidemia, raised blood pressure, and insulin resistance (17). While the definition and criteria of metabolic syndrome differ in each organization (9), obesity and abnormal insulin resistance/glucose intolerance are widely accepted as key risk factors for diabetes mellitus and cardiovascular disease (2). Although some clinical studies have shown that higher body mass index is associated with lower mortality in heart failure (HF) and acute myocardial infarction (MI), also known as the “obesity paradox” (10), insulin resistance, which is present in the majority of people with metabolic syndrome, is strongly associated with heart failure (19). In fact, high-fat diet (HFD)-induced obese mouse models exhibit insulin resistance that is accompanied by cardiac dysfunction (38). Studying the effects of metabolic syndrome on heart failure is an increasingly important and relevant public health issue, as rising rates of obesity and physical inactivity are increasing the prevalence of metabolic syndrome worldwide (2). Therefore, finding potential therapeutic targets for preventing cardiovascular disease in metabolic syndrome is essential to reduce the increased risk of HF morbidity linked with metabolic syndrome.

Mechanistic target of rapamycin (mTOR), a serine/threonine protein kinase, is a key downstream effector of IGF-1 signaling and serves as a critical regulator of cell growth, metabolism, and cell survival (28, 44). mTOR forms two functionally and structurally distinct complexes, mTORC1 and mTORC2 (7, 44). mTORC1 is rapamycin sensitive and phosphorylates p70S6K and 4E-binding protein 1. In contrast, mTORC2 is the kinase responsible for the phosphorylation and activation of Akt (44). Our previous study (5), which used transgenic mice (Tg) with cardiac-specific overexpression of mTOR (mTOR-Tg mice), demonstrated that cardiac mTOR is sufficient to protect the heart against ischemia-reperfusion (I/R) injury in both in vivo and ex vivo models (5). We confirmed that both mTORC1 and mTORC2 are almost equally activated in mTOR-Tg mice (45).

Previous reports have shown that mTOR inhibition by everolimus (a derivative of rapamycin) protected the heart in an in vivo MI model (without reperfusion) (11) and ex vivo I/R model (24). On a related note, more reports have indicated that mTORC1 signaling through p70S6K negatively regulates Akt by inhibiting insulin-receptor substrate-1 (18). This negative feedback loop accounts for the observed activation of Akt and inhibition of mTORC1 by either rapamycin treatment (28) or overexpression of proline-rich Akt substrate of 40 kDa (PRAS40), which binds to and inhibits mTORC1 (49). A recent study (48), which overexpressed PRAS40 in the heart using adeno-associated viral gene transfer, showed that PRAS40 overexpression activates mTORC2 and protects the heart in HFD-induced obese mice. The activity of mTOR and its protective effects in models of obesity could be due to its known role as a key mediator of energy metabolism in many organs, including the heart. However, the cardioprotective role of mTOR itself has not been studied in the context of obesity.

In the present study, we examine the effect of mTOR overexpression on ex vivo cardiac function and in vivo I/R injury in the context of HFD-induced obese mice. Our results demonstrate that cardiac mTOR preserves cardiac function...
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after I/R in both obese mice and lean mice and that this cardioprotection is accompanied by attenuated necrosis and inflammatory response caused by I/R injury.

MATERIALS AND METHODS

Animal models. Animal experiments in this study were approved by the Institution Animal Care and Use Committees of the University of Hawaii (Honolulu, HI). This investigation conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Tg mice expressing hemagglutinin-tagged wild-type (WT) rat mTOR under the direction of the murine α-myosin heavy chain promoter have been previously described in detail (45). Line 4 male (mTOR-Tg) mice, in which mTOR expression was about threefold higher than littermate controls (WT mice), (45), were used for these experiments. Male WT and mTOR-Tg mice at 6 wk of age were fed a HFD for 12 wk and weighed once a week until they reached 18 wk of age. At 6 wk of age, mice were placed on pellets of either a normal chow diet (NCD) consisting of 24.7% energy from protein, 63.4% carbohydrate, and 4.6% fat (PicoLab rodent diet 5053, 339.5 kcal/100 g, TestDiet, Richmond, IN) or a HFD consisting of 14.9% energy from protein, 26.0% carbohydrate, and 59.0% fat (S3282, 549.0 kcal/100 g, Bio-Serv, Frenchtown, NJ) for 12 wk. Previous studies have demonstrated that a 60% fat diet induces insulin resistance as well as obesity and creates a reasonable model for studying pathophysiological features of the cardiovascular system in diet-induced obesity (8, 25, 41). To evaluate HFD-induced glucose intolerance and insulin resistance, we performed glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) after 12 wk of NCD or HFD feeding as previously demonstrated (4, 36). Mice were fasted for 16 h before receiving an intraperitoneal administration of 1.5 g glucose/kg body wt in saline. Blood samples were collected from the tail vein at 0, 10, 30, 60, and 120 min in heparinized microcapillary tubes, and plasma glucose and insulin levels were determined from those samples. An ITT was performed on mice after 1 h of fasting. Animals were intraperitoneally injected with 1.0 U insulin/kg body wt in saline. Blood samples were drawn from the tail vein at 0, 10, 30, 60, and 120 min for the measurement of plasma glucose levels. Plasma glucose levels in blood samples collected from tail veins were determined using a commercially available glucose meter (OneTouch Ultra blood glucose meter, LifeScan, Milpitas, CA). After the isolation of plasma from blood samples, insulin levels were determined by ELISA (Merckodia, Winston Salem, NC). To evaluate the degree of insulin resistance, values were calculated using the following homeostatic model of assessment of insulin resistance (HOMA-IR) formula: fasting glucose (in mg/dl) × fasting insulin (in μU/ml)/405.

Ex vivo I/R in Langendorf perfused hearts. WT and mTOR-Tg mice were subjected to an ex vivo Langendorf perfusion model as previously described (5, 35). After retrograde perfusion was established at a constant pressure (80 mmHg), hearts were perfused with modified Krebs-Henseleit buffer (11 mM glucose, 118 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, and 0.5 mM EDTA) equilibrated with 95% O2-5% CO2 at 37°C to yield a pH of 7.4. A water-filled balloon catheter was introduced into the left ventricle (LV) to record LV pressure (PowerLab, AD Instruments, Denver, CO). We measured a volume of the coronary sinus effluent in the collected perfusate to determine the coronary flow rate. For the ex vivo I/R model, hearts were perfused for 15 min, and the flow was then eliminated for 20 min followed by reperfusion for 40 min. The peak ischemic contracture during the 20-min ischemia period was determined by the alteration of LV end-diastolic pressure, as previously reported (39).

In vivo I/R. Mice were subjected in vivo I/R as previously described (5). Mice were anesthetized by isoflurane. After mice were intubated and ventilated, a left thoracotomy was performed. The left anterior descending coronary artery was ligated with 7-0 silk sutures. After 30 min of occlusion (ischemia), the ligature around the left anterior descending coronary artery was released, and reperfusion was visually confirmed. We evaluated cardiac function in conscious mice with transthoracic echocardiography (Vevo 2100 Imaging system, Visual Sonics, Toronto, ON, Canada) using a MS400 transducer (18–38 MHz). M-mode images used for measurements were taken at the papillary muscle level (45). To determine fibrotic changes after I/R, paraffin-embedded heart sections were stained with Masson’s trichrome (American MasterTech, Lodi, CA). The fibrotic area was determined by ImageJ software (5).

Western blot analysis. Hearts were harvested, snap frozen, and crushed in liquid nitrogen. Tissue was homogenized in cold lysis buffer (Cell Signaling, Danvers, MA) as previously described (5, 45). Protein concentrations were measured by the Bradford method (BioRad, Hercules, CA). SDS-PAGE was performed under reducing conditions on 4–20% gradient gels (Bio-Rad). Blots were incubated with primary antibodies for 18–20 h at 4°C. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody, and signals were detected using enhanced chemiluminescence (Cell Signal). Primary antibodies to hemagglutinin (12CA5, Roche, Indianapolis, IN), S6 (Cell Signaling), phospho-S6 (Ser235/244, Cell Signaling), Akt (Cell Signaling), phospho-Akt (Ser473, Cell Signaling), mTOR (Cell Signaling), growth differentiation factor 15 (GDF15; Bio-Rad), Abcam, Cambridge, MA), and light chain 3 (LC3; Novus, Littleton, CO) were used for immunoblot analysis.

Biological analysis in ex vivo perfused hearts. Enzyme activities of creatine kinase (CK) and lactate dehydrogenase (LDH) were determined in the effluent collected at baseline and 40 min of reperfusion by enzyme activity kits as previously described (CK: BioAssay Systems, Hayward, CA; LDH: Cayman Chemical, Ann Arbor, MI) (5).

Quantitative real-time PCR. Accumulation of PCR products was monitored in real time, and cycle threshold (Ct) values were determined with a 7900HT Fast Real-Time System (Applied Biosystems, Foster City, CA). Relative changes in gene expression were determined using the ΔΔCt method with normalization to β-actin. Quantitative real-time PCR was performed with the following sets of primers: IL-6, forward 5′-AGAGGAGTGCTGTAAGGACAA-3′ and reverse 5′-GAATTACCACACTAGGTGCT-3′; IL-β, forward 5′-CCTTCAGGATAGGACATGAG-3′ and reverse 5′-GTGCCA-CACACCAGGATTATC-3′; TNF-α, forward 5′-AGCAACCA-3′ and reverse 5′-GCTGGCACACTAGTGGTGT-3′; monocyte chemotactic protein-1, forward 5′-ATCCATGAGGAGGAGGACCA-3′ and reverse 5′-ATCGTCA-CCACACCGAGGTTAC-3′; macrophage inflammatory protein-1α, forward 5′-ACCTGCTCAACATCATGAGG-3′ and reverse 5′-AGTTGAGCTATGCGGTTG-3′; and TNF-β, forward 5′-ATGCTGACAAATCAGGACCA-3′ and reverse 5′-GTCGTTAGTGGGTTG-3′.

Statistical analysis. Data are presented as means ± SE. Group differences were analyzed by a two-tailed Student’s or Welch’s t-test. For multiple comparisons, two-way ANOVA was used. For all analyses, P values of <0.05 were considered significant.

RESULTS

Effects of HFD on body weight gain, glucose intolerance, and insulin resistance in WT and mTOR-Tg mice. Both WT and mTOR-Tg mice fed a HFD for 12 wk exhibited an ∼60% increase in body weight compared with mice fed a NCD (P < 0.001 for both groups; Fig. 1A). Fasting and fed plasma glucose levels were higher in HFD-fed mice than in NCD-fed mice (Fig. 1B). GTTs and ITTs clearly demonstrated HFD-induced glucose intolerance and insulin resistance in both WT
Fig. 1. High-fat diet (HFD)-induced obesity, glucose intolerance, and insulin resistance are comparable between wild-type (WT) and mechanistic target of rapamycin (mTOR)-overexpressing transgenic (mTOR-Tg) mice. A: body weight changes during HFD feeding (n = 36–40 mice/group). B: 16-h fasting or fed plasma glucose levels [n = 24 WT mice fed a normal chow diet (WT-NCD mice), 24 mTOR-Tg fed a NCD (mTOR-Tg-NCD mice), 22 WT mice fed a HFD (WT-HFD mice), and 22 mTOR-Tg mice fed a HFD (mTOR-Tg-HFD mice)]. C and D: plasma glucose and insulin levels during glucose tolerance tests. E: plasma glucose levels during insulin tolerance tests. F: homeostatic model of assessment of insulin resistance (HOMA-IR) calculated with the formula described in MATERIALS AND METHODS.*$P < 0.05, **P < 0.01, and ***P < 0.001, HFD vs. NCD (by two-way ANOVA or Student’s t-test).

Table 1. Baseline cardiac function in ex vivo perfused hearts isolated from NCD-fed or HFD-fed WT and mTOR-Tg mice

<table>
<thead>
<tr>
<th></th>
<th>WT NCD-Fed Group</th>
<th>mTOR-Tg NCD-Fed Group</th>
<th>WT HFD-Fed Group</th>
<th>mTOR-Tg HFD-Fed Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice/group</td>
<td>24</td>
<td>28</td>
<td>24</td>
<td>26</td>
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<tr>
<td>LV systolic pressure, mmHg</td>
<td>133.6 ± 6.3</td>
<td>134.8 ± 4.2</td>
<td>163.7 ± 7.7**</td>
<td>160.0 ± 7.0**</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>3.60 ± 0.43</td>
<td>4.83 ± 0.48</td>
<td>4.92 ± 0.43</td>
<td>4.94 ± 0.52</td>
</tr>
<tr>
<td>LV developed pressure, mmHg</td>
<td>130.0 ± 6.2</td>
<td>130.0 ± 4.2</td>
<td>158.8 ± 7.3**</td>
<td>154.9 ± 7.1**</td>
</tr>
<tr>
<td>LV dP/dt_{max}, mmHg/s</td>
<td>5.356 ± 314</td>
<td>5.476 ± 239</td>
<td>7.183 ± 325**</td>
<td>6.836 ± 380**</td>
</tr>
<tr>
<td>LV dP/dt_{min}, mmHg/s</td>
<td>-2.937 ± 141</td>
<td>-2.959 ± 107</td>
<td>-3.822 ± 107**</td>
<td>-3.621 ± 162**</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>308.1 ± 8.1</td>
<td>299.5 ± 9.4</td>
<td>285.8 ± 14.7</td>
<td>277.5 ± 11.8</td>
</tr>
<tr>
<td>Coronary flow, ml/min</td>
<td>3.14 ± 0.22</td>
<td>3.19 ± 0.25</td>
<td>4.23 ± 0.18**</td>
<td>3.98 ± 0.20*</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>139.67 ± 5.4</td>
<td>134.00 ± 3.9</td>
<td>161.92 ± 3.86**</td>
<td>150.65 ± 3.36**†</td>
</tr>
<tr>
<td>Heart weight/tibia length, mg/mm</td>
<td>6.23 ± 0.24</td>
<td>5.95 ± 0.16</td>
<td>7.23 ± 0.16**</td>
<td>6.78 ± 0.15***†</td>
</tr>
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Values are means ± SE. WT, wild-type mice; NCD, normal chow diet; mTOR-Tg, mechanistic target of rapamycin-overexpressing transgenic mice; HFD, high-fat diet. *$P < 0.05, **P < 0.01, and ***P < 0.001, NCD vs. HFD; †$P < 0.05, WT vs. mTOR-Tg groups (by Student’s t-test).
than in the NCD-fed group in both strains, accompanied by increases in coronary flow and heart weight (Table 1). These findings suggest that the HFD feeding increased contractile function. Whereas there was no difference in cardiac function between WT and mTOR-Tg mice on the same diet, in the HFD-fed group, the average heart weight in WT mice was higher than that of mTOR-Tg mice. Taken together, the effect of HFD on baseline cardiac function was comparable between WT and mTOR-Tg mice.

**Overexpression of mTOR prevents cardiac dysfunction after ex vivo I/R in hearts from HFD-fed mice.** We examined cardiac functional recovery in all four mouse groups using ex vivo I/R models. In a manner consistent with our previous report (5), LVDP in mTOR-Tg NCD-fed hearts was significantly higher at all reperfusion time points compared with WT NCD-fed hearts ($P < 0.01$; Fig. 2A). End-point LVDP recovery in mTOR-Tg NCD-fed hearts was $\sim30\%$ higher compared with WT NCD-fed hearts ($P < 0.05$; Fig. 2B). LVDP during reperfusion and end-point LVDP recovery were strikingly reduced by HFD feeding compared with NCD feeding in WT hearts (LVDP during reperfusion: WT NCD-fed vs. WT HFD-fed groups, $P < 0.001$; LVDP recovery: $41.90 \pm 3.85\%$ in the WT NCD-fed group vs. $14.50 \pm 4.50\%$ in the WT HFD-fed group, $P = 0.001$; Fig. 2, A, top, and B). mTOR-Tg HFD-fed hearts showed significantly higher functional recovery compared with WT HFD-fed hearts ($P < 0.05$; Fig. 2, A, top, and B). LV $dP/dt_{\text{max}}$ and LV $dP/dt_{\text{min}}$ during I/R experiments showed results consistent to the LVDP results (Fig. 2A, middle). These data indicate that the overexpression of cardiac mTOR prevents HFD-induced deterioration of cardiac function during ex vivo I/R.

Ischemic contracture after ischemia is a crucial determinant of I/R and is caused by cytosolic Ca$^{2+}$ overload and ATP depletion (26, 46). In WT hearts, HFD feeding significantly increased ischemic contracture during 20-min ischemia compared with NCD feeding (peak ischemic contracture: $69.47 \pm 2.65$ mmHg in the WT NCD-fed group vs. $83.9 \pm 4.50$ mmHg in the WT HFD-fed group, $P < 0.01$; Fig. 2, A, bottom, and C). mTOR-Tg hearts exhibited less ischemic contracture than WT hearts in both NCD-fed and HFD-fed groups (WT NCD-fed vs. mTOR-Tg NCD-fed groups, $P < 0.01$; WT HFD-fed vs. mTOR-Tg HFD-fed groups, $P < 0.01$; Fig. 2, A, bottom, and C). These results suggest that overexpression of mTOR suppresses ischemic contracture.

**Cardiac mTOR suppresses necrosis in ex vivo I/R with HFD-fed hearts.** Since necrosis is a key factor in the pathogenesis of I/R injury (51), we measured CK and LDH concentrations in the effluent during reperfusion as markers of cardiomyocyte necrosis after I/R. As shown in our previous report (5), overexpression of mTOR attenuated both CK and LDH release after I/R in NCD-fed groups (Fig. 3, A and B). The administration of a HFD exacerbated I/R-induced CK and LDH release after I/R in NCD-fed groups (Fig. 3, A and B). The administration of a HFD exacerbated I/R-induced CK and LDH release in both strains (CK: WT NCD-fed vs. WT HFD-fed groups, $P < 0.001$, and mTOR-Tg NCD-fed vs. mTOR-Tg HFD-fed groups, $P < 0.01$; LDH: WT NCD-fed vs. WT HFD-fed groups, $P < 0.001$, and mTOR-Tg NCD-fed vs. mTOR-Tg HFD-fed groups, $P < 0.01$; Fig. 3, A and B). As we expected, both CK and LDH concentrations were significantly lower in effluent samples from mTOR-Tg HFD-fed hearts compared with those of WT HFD-fed hearts after I/R (CK: WT HFD-fed vs. mTOR-Tg HFD-fed groups, $P < 0.05$; LDH: WT HFD-fed...
high, especially in HFD conditions, it was difficult to
ylation levels of both S6 and Akt in hearts after I/R were
HFD-fed mice after I/R (Fig. 4). However, since phosphor-
phosphorylation between WT HFD-fed and mTOR-
in WT mice, whereas no difference was observed in S6
phosphorylation was higher after I/R in mTOR-Tg mice than
compared with baseline conditions. In HFD conditions, Akt
both S6 and Akt were significantly increased in all groups
with baseline (Fig. 4). In I/R hearts, phosphorylation levels of
the level of mTOR expression in mTOR-Tg hearts compared
vs. baseline (Fig. 4). I/R increased phosphorylation
mTOR-Tg mice. The HFD increased baseline phosphoryla-
tion of Akt also increased in HFD-fed mice, although it was not
statistically significant (Fig. 4). 

Effects of HFD on the mTOR signaling pathway in WT and mTOR-Tg mice. The HFD increased baseline phosphorylation of S6 in both WT and mTOR-Tg mice (Fig. 4). Phosphorylation of Akt also increased in HFD-fed mice, although it was not statistically significant (Fig. 4). I/R increased phosphorylation of both S6 and Akt compared with the baseline phosphorylation in all genotype and diet conditions, whereas I/R decreased the level of mTOR expression in mTOR-Tg hearts compared with baseline (Fig. 4). In I/R hearts, phosphorylation levels of both S6 and Akt were significantly increased in all groups compared with baseline conditions. In HFD conditions, Akt phosphorylation was higher after I/R in mTOR-Tg mice than in WT mice, whereas no difference was observed in S6 phosphorylation between WT HFD-fed and mTOR-Tg HFD-fed mice after I/R (Fig. 4). However, since phosphorylation levels of both S6 and Akt in hearts after I/R were high, especially in HFD conditions, it was difficult to
identify whether mTOR overexpression made a difference in activation between mTORC1 and mTORC2 after I/R.

Cardiac mTOR attenuates the production of proinflammatory factors in I/R injury. To assess the effect of mTOR in the inflammatory response after I/R, we measured levels of IL-6, IL-1β, TNF-α, monocyte chemotactic protein-1, and macrophage inflammatory protein-1α. In a manner consis-
Fig. 3. Overexpression of cardiac mTOR prevents cardiac injury after ex vivo transient ischemia in HFD hearts. A and B: activities of creatine kinase (CK; A) and lactate dehydrogenase (LDH; B) in the effluent collected during the reperfusion period. To determine enzyme activities immediately after ex vivo I/R injury, effluents from hearts exposed to either 20 or 40 min of global ischemia were collected at control perfusion (baseline) and after 40-min reperfusion (I/R). n = 14 WT-NCD mice, 5 mTOR-Tg-NCD mice, 13 WT-HFD mice, 15 mTOR-Tg-HFD mice. *P < 0.05 and ***P < 0.001, NCD vs. HFD; †P < 0.05 and ††P < 0.01, WT vs. mTOR-Tg mice; †††P < 0.01 and ††††P < 0.001, baseline vs. I/R (by Student’s t-test).

Fig. 4. Overexpression of cardiac mTOR induces functional activation of both mTORC1 and mTORC2 in post-I/R hearts. A: representative immunoblots of mTOR signaling molecules in hearts subjected to the ex vivo Langendorff perfusion model. Baseline hearts were harvested after 15 min of equilibration perfusion ex vivo. I/R hearts were harvested after a course of baseline conditions followed by 20-min ischemia and then 40-min reperfusion. Immunoblot analysis was performed with the indicated antibodies. Blots are representative of six independent experiments. Densitometric quantitative analyses of mTOR (A), phospho-S6 (C), and phospho-Akt (D) were normalized to baseline levels of WT-NCD hearts in each experiment. n = 6 baseline hearts and 12 I/R hearts. *P < 0.05 and ***P < 0.001, NCD vs. HFD; †P < 0.05, ††P < 0.01, and †††P < 0.001, WT vs. mTOR-Tg hearts; †P < 0.05, ††P < 0.01, and †††P < 0.001, baseline vs. I/R (by Student’s t-test).

Fig. 5. Overexpression of mTOR does not affect autophagic activity in a NCD nor HFD. A: representative immunoblots of light chain 3 [LC3; LC3-I (top) and LC3-II (bottom)] and GAPDH levels in hearts subjected to the ex vivo Langendorff perfusion model. Hearts were harvested after I/R as described in Fig. 4. B: densitometric analysis of LC3-II levels normalized to GAPDH. n = 6 for all groups.
tent with our previous report (5), expression levels of these proinflammatory cytokines and chemokines in NCD-fed mouse hearts were significantly increased in I/R hearts compared with baseline hearts in both strains, and mTOR overexpression suppressed the expression of these proinflammatory factors compared with WT mice (Fig. 6). Interestingly, while mTOR overexpression also suppressed cardiac expression of proinflammatory factors in response to the HFD, HFD treatment did not significantly affect them in WT and mTOR-Tg hearts under baseline or I/R conditions (Fig. 6).

Previous reports have shown that cardiac GDF15, a member of the transforming growth factor-β superfamily, plays an important role in cardioprotection against myocardial ischemia (23) and pathological hypertrophy (52). Since mTORC2 regulates GDF15 expression in the heart (12), we examined GDF15 mRNA expression in the hearts of WT and mTOR-Tg mice fed either NCD or HFD. The expression level of GDF15 was significantly higher in mTOR-Tg hearts compared with WT hearts across all experimental groups (Fig. 6, bottom right). I/R significantly increased GDF15 expression in both WT NCD-fed and WT HFD-fed hearts compared with baseline levels (P < 0.01; Fig. 6, bottom right). To confirm our findings at the mRNA level, we measured baseline protein levels of GDF15 in the hearts of NCD-fed mice. The level of GDF15 protein in the heart was ~40% higher in mTOR-Tg mice compared with WT mice (Fig. 7). These data suggested that mTOR overexpression increased GDF15 in both NCD and HFD conditions.

Overexpression of mTOR rescues the detrimental effects of HFD-induced obesity on post-I/R cardiac function and LV remodeling in vivo. To confirm the ex vivo cardio protective effect of mTOR against I/R under conditions of HFD-induced obesity, we measured cardiac function and LV mass in WT and mTOR-Tg mice fed either NCD or HFD. The expression level of GDF15 was significantly higher in mTOR-Tg hearts compared with WT hearts across all experimental groups (Fig. 6, bottom right). I/R significantly increased GDF15 expression in both WT NCD-fed and WT HFD-fed hearts compared with baseline levels (P < 0.01; Fig. 6, bottom right). To confirm our findings at the mRNA level, we measured baseline protein levels of GDF15 in the hearts of NCD-fed mice. The level of GDF15 protein in the heart was ~40% higher in mTOR-Tg mice compared with WT mice (Fig. 7). These data suggested that mTOR overexpression increased GDF15 in both NCD and HFD conditions.
obesity, we performed in vivo I/R on WT and mTOR-Tg mice after 12 wk of HFD feeding, as previously described (5). Echocardiography showed that systolic function in both WT and mTOR-Tg groups was reduced 1 wk after I/R (Fig. 8, A and B). Fractional shortening in the mTOR-Tg group was higher than in the WT group, suggesting again that mTOR overexpression protects hearts during the acute phase after I/R, which is consistent with our previous report (5). The HFD further exacerbated the reduction in fractional shortening caused by in vivo I/R in both WT and mTOR-Tg mice (WT groups: WT NCD-fed vs. WT HFD-fed groups, P < 0.001; and mTOR-Tg groups: mTOR-Tg NCD-fed vs. mTOR-Tg HFD-fed groups, P < 0.05; Fig. 8B). mTOR-Tg mice exhibited higher fractional shortening than WT mice after in vivo I/R, and this difference was observed in both types of dietary conditions (NCD: WT vs. mTOR-Tg mice, P < 0.01; and HFD: WT vs. mTOR-Tg mice, P < 0.01; Fig. 8B). We also evaluated myocardial fibrosis in the heart 1 wk after I/R. The result was consistent with the changes observed in cardiac function, and overexpression of mTOR reduced fibrosis in post-I/R hearts compared with WT mice in both NCD and HFD feeding conditions (Fig. 8, C and D). As previously described (5, 21, 27), myocardial scarring in the heart after in vivo I/R was located along circumferential myofibers in the midcardium and extended from the initial infarct to the remote zone (Fig. 8C). Our results confirm that HFD-induced obesity worsens the decreased cardiac function observed in vivo I/R injury and suggest that overexpression of mTOR lessened the detrimental effects of a HFD, at least in part, by suppressing adverse LV remodeling.

**DISCUSSION**

In the present study, we demonstrate that diet-induced obesity deteriorates post-I/R cardiac functional recovery in both ex vivo Langendorff perfused hearts and in vivo coronary ligation model. With these models, we also show that overexpression of cardiac mTOR protects the heart against I/R injury in both obese and lean mice. We also observed that overexpression of mTOR suppresses I/R-induced inflammation and necrosis in ex vivo I/R models. Our in vivo I/R models show that in both obese and lean mice, mTOR overexpression prevents extension of myocardial scarring, which is a key pathophysiological event in adverse LV remodeling due to I/R injury (21). We propose that increased mTOR expression inhibits post-I/R myocardial scarring, thus resulting in preserved cardiac function in diet-induced obese mice.

Previous large-scale clinical studies suggest that diabetes is a powerful risk factor for death and heart failure after an acute MI (1, 33, 34). Metabolic syndrome, which includes prediabetes and diabetes (17), is recognized as a predictor of heart failure in patients with acute MI (50). An animal study (47) using HFD-induced obese mice has shown that obesity further deteriorates cardiac function in I/R injury. Our results are consistent with those findings, as we observed that cardiac function recovery after temporary ischemia in ex vivo perfused hearts was worse in HFD-induced obese mice than in lean mice, and this worse outcome was accompanied by an increased release of necrotic markers CK and LDH in obese mice compared with lean mice. In vivo models of I/R injury have also shown that diet-induced obesity deteriorated cardiac function after I/R compared with lean mice. Previously, we reported that mTOR overexpression protects the heart against I/R injury in both in vivo and ex vivo models using the same Tg mice as in the present study (5). The present study demonstrates that mTOR overexpression preserved cardiac function and prevented cardiac injury in both in vivo and ex vivo models of I/R injury in both obese and lean mice. This may initially seem contrary to a recent report (42) that demonstrated that mTORC1 inhibition by either rapamycin or partial mTOR deletion protected the heart against MI (without reperfusion) in diet-induced obese mice. However, these findings might be indicative of activation of phosphatidylinositol 3-kinase/Akt

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**Fig. 8.** Overexpression of cardiac mTOR preserves cardiac function after in vivo transient ischemia in HFD hearts. A: representative M-mode images of operated WT-NCD, mTOR-Tg-NCD, WT-HFD, and mTOR-Tg-HFD mice at baseline and 1 wk after I/R surgery. B: mean scores for fractional shortening (FS; in %) at 1 wk after surgery. C: representative Masson’s trichrome staining after 1 wk of surgery. D: quantitative analysis of interstitial fibrosis detected by Masson’s trichrome staining. n = 12 WT-NCD hearts, 12 mTOR-Tg-NCD hearts, 15 WT-HFD hearts, and 10 mTOR-Tg-HFD hearts. *P < 0.05, **P < 0.01, and ***P < 0.001, NCD vs. HFD; †P < 0.05 and ††P < 0.01, WT vs. mTOR-Tg hearts; †††P < 0.001, baseline vs. I/R (by Student’s t-test).
signaling by suppression of negative feedback inhibition of insulin-receptor substrate-1 by mTORC1 (18). In fact, another study using mTOR-binding protein PRAS40, which inhibits mTORC1, showed that PRAS40 overexpression activates mTORC2 and exhibits cardioprotection in HFD-induced obese mice. In our Tg mice, overexpression of WT mTOR activates both mTORC1 and mTORC2 signaling pathways (5, 45). In our study, I/R increased phosphorylation levels of both S6 and Akt in all groups, especially in the HFD-fed groups. This was likely affected by the hyperinsulinemia observed in both HFD-fed groups (Fig. 1), and the elevated insulin levels probably activated both kinases. Whereas no statistical difference in S6 phosphorylation was observed between WT HFD-fed and mTOR-Tg HFD-fed mice after I/R, Akt phosphorylation was higher in mTOR-Tg mice than WT mice under HFD conditions. Our results, combined with the previous reports discussed above, suggest that mTORC2 activation might be a key factor for cardioprotection against I/R injury under both obese conditions. However, since we did not selectively manipulate the mTORC2 signaling pathway in the present study, further experiments are required to demonstrate the cardioprotective role of mTORC2 under obese conditions.

Autophagy is a catabolic cellular process mediated by lysosomes and plays an important role in cellular homeostasis in many organs, including the heart (14, 29). The mTORC1 axis is a potential antiapoptotic response that regulates the UNC-51-like kinase complex (14). In our study, mTOR overexpression appeared to slightly suppress LC3-II expression, but this was not statistically significant. A previous report (43) has shown that HFD-induced obesity exhibits autophagy dysregulation. In contrast, LC3-II expression in our HFD-fed mice was comparable to that of NCD-fed mice. Since mTOR overexpression did not significantly affect baseline levels of LC3-II, it is unlikely that the protective effects of mTOR are mediated through autophagic activity. Another detection system, such as LC3-green fluorescent protein mice (32), would be necessary to define the role of mTOR-regulated autophagy in metabolic syndrome.

In a recent report (21), we used three-dimensional images rendered from multiple histological sections from the heart after in vivo I/R injury to show that myocardial scarring after I/R, but not straight MI (no reperfusion), extends along myofibers rather than coronary arteries. In the present study, histological assays in in vivo I/R models showed that fibrotic scars extended along myofibers in the midcardium and were larger in HFD-induced obese mice than in lean mice and that mTOR overexpression prevented the extension of myocardial scarring along myofibers compared with WT hearts in both feeding conditions. This pattern of scarring is similar to that of contraction band necrosis, which is observed in reperfusion after ischemia (31). Proposed pathogeneses of contraction band necrosis are shared with hypercontracture, including Ca^{2+} overload and ATP resynthesis (40). We observed that in ex vivo I/R models, myocardial contracture during diastole in both late ischemia and reperfusion phases was higher in HFD-fed mice compared with NCD-fed mice and that mTOR overexpression suppressed the level of contracture in both feeding conditions. While prolonged and/or excess hypercontracture seems to be associated with band necrosis (40), the mechanisms of ischemic contracture and hypercontracture in I/R injury have not been characterized well. In the present study, HFD-fed mTOR-Tg mice exhibited less ischemic contracture, better cardiac recovery, and less myocardial injury compared with HFD-fed WT mice. The difference in myocardial contracture in mTOR-Tg mice may contribute to their better functional recovery after I/R, especially in obese mice.

Chronic inflammation accounts for pathophysiological features of obesity-induced insulin resistance (15, 53). It is also known that cardiomyocytes are key resources of cytokines and chemokines (6). However, our results demonstrated that there was no significant difference in expression levels of I/R-induced cytokines and chemokines between the hearts of NCD and HFD mice, and this trend held true for both WT and mTOR-Tg mice. A possible explanation for this lack of difference is that 1–3 days after the onset of MI in vivo, leukocytes are recruited and accumulated in the infarct region (13). It is likely that activation of an inflammatory response was mainly triggered by circulating leukocytes rather than the cells of the heart, including cardiac fibroblasts and cardiomyocytes. We have previously reported that overexpression of cardiac mTOR suppresses the production of proinflammatory cytokines and chemokines in both transverse aortic constriction-induced cardiac hypertrophy and I/R (5, 45). In the present study, we also observed that mTOR overexpression suppressed proinflammatory cytokine and chemokine mRNA expression in I/R injury compared with WT hearts in both feeding conditions. Cardiac mTOR-mediated downregulation of inflammatory cytokine production from cardiomyocytes might contribute to reduced LV remodeling after I/R. However, it is known that the magnitude of LV remodeling is directly proportional to the initial infarct size after acute MI (30). Further experiments are required to determine the importance of suppression of the inflammatory response during mTOR-mediated cardioprotection against I/R injury and subsequent LV remodeling in obese mice.

Previous studies using genetic mouse models demonstrated that GDF15 protects the heart against pathological hypertrophy (54) and heart failure resulting from MI (22, 23). Our data show that overexpression of cardiac mTOR increased expression levels of GDF15 mRNA and protein in baseline conditions. Expression of GDF15 is regulated by activating transcription factor 4, which is downstream of the PKR-like endoplasmic reticulum kinase-eukaryotic translation initiation factor-2α pathway (3, 20). This pathway could overlap with the results of another study (37), in which TSC deletion caused mTOR activation and increased phosphorylation of PKR-like endoplasmic reticulum kinase. Based on those results and the increased GDF15 we observed, it is likely that cardiac mTOR may regulate the expression of GDF15 and its signaling pathway. Further experiments will be needed to elucidate and define the role of cardiac mTOR in regulating GDF15.

In conclusion, we demonstrated that overexpression of cardiac mTOR prevented the detrimental effects of fat-induced obesity on cardiac dysfunction in ex vivo and in vivo I/R and that cardiac mTOR overexpression was accompanied by suppression of proinflammatory cytokine production and necrosis. The rising rate of morbidity from heart failure after acute MI is further exacerbated by the presence of metabolic syndrome, and, thus, the effective treatment of both diseases remains a critical issue in the clinic. Interestingly, recent reports have shown that several mTOR-stabilizing proteins control the level of mTOR expression. Understanding the mechanisms of mTOR.
protein regulation and further studying the cardioprotective effects of mTOR against I/R injury could provide an important clue for therapies to prevent HF in the midst of metabolic syndrome.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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