Heart Failure

Cardiac Raptor Ablation Impairs Adaptive Hypertrophy, Alters Metabolic Gene Expression, and Causes Heart Failure in Mice

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Background—Cardiac hypertrophy involves growth responses to a variety of stimuli triggered by increased workload. It is an independent risk factor for heart failure and sudden death. Mammalian target of rapamycin (mTOR) plays a key role in cellular growth responses by integrating growth factor and energy status signals. It is found in 2 structurally and functionally distinct multiprotein complexes called mTOR complex 1 (mTORC1) and mTORC2. The role of each of these branches of mTOR signaling in the adult heart is currently unknown.

Methods and Results—We generated mice with deficient myocardial mTORC1 activity by targeted ablation of raptor, which encodes an essential component of mTORC1, during adulthood. At 3 weeks after the deletion, atrial and brain natriuretic peptides and β-myosin heavy chain were strongly induced, multiple genes involved in the regulation of energy metabolism were altered, but cardiac function was normal. Function deteriorated rapidly afterward, resulting in dilated cardiomyopathy and high mortality within 6 weeks. Aortic banding–induced pathological overload resulted in severe dilated cardiomyopathy already at 1 week without a prior phase of adaptive hypertrophy. The mechanism involved a lack of adaptive cardiomyocyte growth via blunted protein synthesis capacity, as supported by reduced phosphorylation of ribosomal S6 kinase 1 and 4E-binding protein 1. In addition, reduced mitochondrial content, a shift in metabolic substrate use, and increased apoptosis and autophagy were observed.

Conclusions—Our results demonstrate an essential function for mTORC1 in the heart under physiological and pathological conditions and are relevant for the understanding of disease states in which the insulin/insulin-like growth factor signaling axis is affected such as diabetes mellitus and heart failure or after cancer therapy. (Circulation. 2011;123:1073-1082.)

Key Words: heart failure ■ hypertrophy ■ myocardial metabolism ■ signal transduction

Although cardiac hypertrophy is a growth response that initially normalizes wall tension, it is associated with an unfavorable outcome: Affected patients are threatened with sudden death or progression to heart failure.1 Much research is therefore aimed at understanding myocardial growth regulation, and in this setting, the insulin-like growth factor/PI3-kinase/Akt signaling cascade has been studied extensively.2,3 Experiments with cultured cardiomyocytes have suggested that downstream of Akt, mammalian target of rapamycin (mTOR) mediates responses to pathological stimuli.4,5 mTOR is an evolutionary conserved Ser/Thr kinase known to control cell growth.6 Nutrient, energy, and growth factor shortage will impair mTOR activity, resulting in diverse effects, including the slowdown of macromolecule synthesis, enhanced autophagy, and activation of nutrient- or stress-responsive transcription factors. mTOR is found in 2 structurally and functionally distinct multiprotein complexes called mTOR complex 1 (mTORC1) and mTORC2. The 2 best-characterized substrates of mTORC1 are S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP), through which mTORC1 regulates cap-dependent protein translation.7 In addition, numerous novel effects downstream of mTORC1, not all related to translational activation, have recently been identified (reviewed elsewhere8–10). For instance, mTORC1 regulates autophagy and membrane trafficking for the delivery of nutrient transporters to the cell surface. mTORC2 controls actin organization and most likely other processes that remain to be elucidated. The

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role of these distinct branches of mTOR signaling in cardiac tissue has not been investigated.

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**Clinical Perspective on p 1082**

Support for a role of mTOR in pathological hypertrophy was provided by studies with rapamycin, which repressed or partially prevented hypertrophy induced by constitutive activation of Akt11 and acute or sustained pressure overload.11–15 However, other studies have challenged the importance of mTOR-mediated protein synthesis and growth. For example, deletion of the ribosomal S6 kinases, direct downstream effectors of mTOR, did not attenuate pathological, physiological, or insulin-like growth factor receptor–induced cardiac hypertrophy.16 Moreover, physiological or pathological hypertrophic growth was not affected in mice expressing a kinase-dead mTOR.17 It should also be noted that although mTORC1 is considered the primary target of rapamycin, long-term rapamycin treatment also inhibits mTORC2 in certain cell types18; on the other hand, rapamycin does not inhibit all mTORC1 functions.19 Therefore, studies using rapamycin systemically have not adequately addressed the relative importance of mTORC1 and mTORC2 in cardiomyocytes.

In the present study, we used inducible cre-loxP recombination to delete raptor, which encodes an essential and specific subunit of mTORC1, selectively from cardiomyocytes. We demonstrate that raptor is required for normal physiological function of the heart and for cardiac adaptation to increased workload. The attenuated mTORC1 activity critically affected cardiac protein and energy metabolism, mitochondrial content, apoptosis, and autophagy and rapidly led to cardiac failure.

**Methods**

**Animal Models**

Mice analyzed in this study were backcrossed to C57BL/6J for 6 to 8 generations and crossed to obtain mice positive for MHC-MerCreMer carrying the wild-type raptor alleles (raptor+/+). Littermates were assigned to the control and knockout groups, with each experimental group consisting of mice derived from at least 4 litters. Intraperitoneal injections of tamoxifen citrate (20 mg/kg; Sigma, St Louis, MO) in 60% PBS/40% ethanol were used to induce excision of raptor. Exposures to pressure overload or exercise started 2 weeks after the last injection. For voluntary exercise, mice were individually housed in cages equipped with a running wheel. Transverse aortic constriction (TAC) was performed and cardiac function was determined with the Vevo 770 Ultrasonograph (VisualSonics, Toronto, ON, Canada) as detailed in the online-only Data Supplement, with the investigator blinded to genetic background and treatment group. All animal experiments were carried out according to guidelines for the care and use of laboratory animals and with approval of the Swiss authorities.

**Statistical Analysis**

Data are presented as mean±SEM. Differences in means between 2 groups were evaluated with unpaired 2-tailed Student t tests and those among multiple groups with 1-way ANOVA followed by Bonferroni posthoc tests. When cardiac function was measured at multiple time points, we used repeated-measures ANOVA. Mortality data were analyzed by the log-rank test. All statistics was performed with GraphPad Prism 4.0 software (GraphPad, San Diego, CA). Values of P<0.05 were considered statistically significant.

**Supplemental Methodology**

Routine procedures for surgery, echocardiography, isolated heart measurements, Western blotting, real-time polymerase chain reaction, histology, and transmission electron microscopy are detailed in the online-only Data Supplement.

**Results**

**Analysis of Cardiac Raptor Knockout Mice at 2 Weeks After Gene Excision**

To study the function of mTORC1 in adult mouse heart, we generated mice homozygous for loxP-flanked raptor exon G20,21 and positive for tamoxifen-inducible Cre recombinase driven by the cardiomyocyte-specific α-myosin heavy chain (MHC) promoter (MHC-MerCreMer/raptorfl/fl).22 Tamoxifen injections at the age of 10 to 11 weeks for 5 days induced Cre-mediated recombination, and Western analysis of protein lysates confirmed that raptor protein was reduced by 69% in cardiac (P=0.004) but not in skeletal muscle (Figure 1A).

Thus, tamoxifen at 20 mg/kg body weight22 was sufficient to induce tissue-specific raptor deletion. Hereafter, we refer to the tamoxifen-treated MHC-MerCreMer/raptorfl/fl mice as raptor-cKO. Figure 1B shows that at 2 weeks after gene excision, raptor deficiency was accompanied by lower phosphorylated 4E-BP1 (61%; P=0.028) and ribosomal protein S6 (67%; P<0.0001) compared with tamoxifen-treated MHC-MerCreMer/raptorfl/+ controls. Nonspecific metabolic effects, previously reported to occur transiently in α-MHC-MerCreMer transgenic mice, peaking at 3 days after high-dose tamoxifen (80 mg/kg)22 were excluded because no differences in expression of metabolic and stress-induced genes existed between the 3 tamoxifen-treated groups: Cre-negative/raptorfl/+, MHC-MerCreMer/raptor+/+, and MHC-MerCreMer/raptorfl/fl (raptor-cKO) mice (Figure 1C). Consistently, ex vivo working heart experiments at 2 weeks after tamoxifen revealed no differences in palmitate (P=0.87) or glucose (P=0.71) oxidation (Figure 1D) and in developed pressure (P=0.96) or cardiac output (P=0.84) (Figure 1E).

Thus, cardiac raptor deletion resulted in reduced levels of phosphorylated 4E-BP1 and S6, but no molecular, metabolic, or functional changes were detected at this early time point.

**Cardiac Raptor Ablation Leads to Acute Dilated Cardiomyopathy in Response to Overload**

Because mTORC1 is known to accelerate protein synthesis via 4E-BP1 and p70S6K and because protein synthesis is an intrinsic feature of growth responses, we tested whether raptor deficiency diminishes cardiac hypertrophy under conditions of pressure overload and, if so, how this affects cardiac function. Raptor-cKO mice and controls were assigned to 2 subgroups for subsequent TAC or sham surgery at 2 weeks after tamoxifen. Echocardiography before surgery showed no difference in cardiac function and geometry between the groups (Table I in the online-only Data Supplement). One week after surgery, the wild-type mice had a significantly thicker left ventricular free wall and septum compared with baseline and compared with values measured in the sham-operated group (the Table). End-systolic and end-diastolic left ventricular internal diameters were unchanged, and cardiac function was preserved in this wild-type
group because ejection fractions and fractional shortening were not affected by TAC (the Table).

In contrast, aortic constriction significantly reduced ejection fraction and fractional shortening in raptor-cKO mice (the Table). This was accompanied by reduced left ventricular posterior wall and septal thickness and an increase in left ventricular internal diameter. Moreover, raptor-cKO mice displayed body weight loss, and postmortem analysis showed reduced epididymal fat pad and gastrocnemius weights (the Table), a cachectic phenotype reminiscent of advanced congestive heart failure. Together, these data showed that raptor-cKO mice rapidly developed dilated cardiomyopathy and severe cardiac dysfunction in response to pressure overload.

**Raptor Knockout Mice Do Not Increase Heart Weight and Cardiomyocyte Cross-Sectional Area After Aortic Constriction, But Atrial and Brain Natriuretic Peptides and β-MHC Are Induced**

To evaluate the development of hypertrophy, we determined the ratios of ventricular weight to tibia length 1 week after TAC (Figure 2A). No differences existed between the sham-operated wild-type and raptor-cKO groups. In TAC-operated wild-type mice, the ratio of ventricular weight to tibia length showed an increase in heart mass of 47% over sham-operated controls. In contrast, raptor-cKO mice demonstrated no hypertrophic growth. The modest increase in size observed in raptor-cKO mice was due to dilatation of the heart rather than an increase in weight (Figure 2B). To assess whether this impaired adaptive response was related to an inability to induce an increase in cardiomyocyte size, we stained cardiac sections with wheat germ agglutinin (Figure 2C). TAC increased cardiomyocyte cross-sectional area in wild-type mice by 50%. In contrast, the cross-sectional area of cardiomyocytes in raptor-cKO mice after TAC was not different from that in sham-operated mice.

Quantitative increases in cardiac mass after pressure overload are usually brought about by the re-expression of a fetal gene program. Already under basal conditions, raptor deficiency reduced α-MHC to 52%, whereas it robustly increased β-MHC, atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) mRNA levels by 12.3-, 25.9-, and 12.2-fold compared with wild-type, respectively. For β-MHC and ANP, these changes were similar to those observed after TAC in wild-type mice (Figure 3A). In raptor-cKO mice subjected to TAC, β-MHC and BNP mRNA increased even further. In contrast, α-skeletal actin was not changed after raptor deletion in sham mice. After TAC, its expression increased in wild-type mice as expected, but this increase was attenuated in the raptor-cKO mice (Figure 3A), suggesting that its induction depends on mTORC1. Along with mRNA levels, protein amounts of ANP and β-MHC were increased in hearts of raptor-cKO mice (Figure 3B and 3C). The observed induction of compensatory genes under baseline conditions indicates that raptor deficiency is a stress stimulus for the heart.

**Raptor Deficiency Alters Mediators of Protein Synthesis and Degradation**

To address the mechanisms behind the observed defects, we analyzed protein extracts by Western blotting. In wild-type mice, TAC significantly increased the cardiac amounts of Akt and mTOR, increases that were absent after raptor deletion (Figure 4A and 4B). Total protein of 4E-BP1, a direct downstream target of mTOR, was also increased after TAC, and the presence of the 2 higher-molecular-weight β- and γ-bands indicated that it was to a large extent phosphorylated (Figure 4A and 4C). In the raptor-cKO mice, a shift toward the lower-molecular-weight α-band indicated reduced 4E-BP1 phosphorylation, which was confirmed for the TAC-operated mice after quantification of the β-band (Figure 4C) or with an antibody to the Thr70 site of 4E-BP1 (Figure 4A). Moreover, although total p70-S6K1 protein was equal for all groups, phosphorylation of its effector S6 was decreased in raptor-cKO hearts compared with pressure-overloaded hearts.
of wild-type mice (Figure 4A and 4C). Interestingly, despite the fact that raptor deficiency was accompanied by reduced total Akt protein, Akt phosphorylation at Thr308 and Ser473 was markedly increased compared with wild-type mice (Figure 4D). Glycogen synthase kinase 3β, a target of Akt, showed strongest phosphorylation at Ser9 in the raptor-cKO mice subjected to sham surgery (Figure 4A and 4D). Importantly, hyperphosphorylation of Akt at Ser473, one of the known downstream targets of mTORC2, indicated selective inhibition of the mTORC1 pathway solely.

Figure 4E shows that muscle atrophy F-box and muscle-specific RING finger protein (MuRF)1 mRNA levels were modestly decreased under baseline conditions, whereas MuRF3 mRNA was significantly higher after TAC in raptor-cKO compared with wild-type mice. Thus, reduced mTORC1 activity affected protein synthesis and degradation pathways.

**Raptor Deficiency Causes a Switch From Fatty Acid to Glucose Oxidation**

Because we observed a reinduction of the fetal gene program without concurrent changes in function under conditions of normal cardiac load at 3 weeks of gene ablation, we followed up on the consequences of raptor deletion at later time points under physiological conditions, ie, in sedentary mice or mice exercising on a voluntary basis in a running wheel. Ejection fractions were normal in nearly all mice up to 3 weeks after raptor deletion but decreased to lower values at 38 days (P<0.01; Figure 5). Raptor-cKO mice started to die during the fifth week after tamoxifen. At that time point, a trend toward increased mortality was observed in exercising (64%, n=14) compared with sedentary (36%, n=11) mice (P=0.097). Surviving sedentary raptor-cKO mice had a mean ejection fraction of 17.3±1.9%, left ventricular posterior wall of 0.60±0.03 and 0.71±0.02 mm, and left ventricular internal diameter of 5.0±0.1 and 4.6±0.1 mm during diastole and systole, respectively. After exercise, similar values were obtained. Thus, the ejection fraction was 18.9±7.5%, the diastolic/systolic left ventricular posterior wall was 0.63±0.01/0.69±0.02 mm, and the diastolic/systolic left ventricular internal diameter was 4.8±0.1/4.4±0.2 mm. These values indicate a significant loss of function culminating with sudden death. None of the wild-type mice died during this period.

**Raptor Deficiency Leads to Cardiac Dysfunction and Mortality Under Physiological Conditions**

Because we observed a reinduction of the fetal gene program without concurrent changes in function under conditions of normal cardiac load at 3 weeks of gene ablation, we followed up on the consequences of raptor deletion at later time points under physiological conditions, ie, in sedentary mice or mice exercising on a voluntary basis in a running wheel. Ejection fractions were normal in nearly all mice up to 3 weeks after...
whether cardiac raptor ablation changes metabolic substrate use. At 2 weeks after tamoxifen, substrate use was normal, but at later time points, palmitate oxidation was decreased to 51% and glucose oxidation increased by 24% above wild-type levels (Figure 6A). In subsequent experiments, we tested whether at 3 weeks after tamoxifen, a time point at which cardiac function was still normal, raptor ablation changed gene expression of factors that regulate substrate use. In the heart, fatty acid oxidation is regulated by estrogen-related receptor α (ERα) and peroxisome proliferator-activated receptor α (PPARα), binding partners of PGC1α. A decrease in ERα to 75% in the raptor-cKO mice did not reach significance, but PPARα and PGC1α were significantly reduced to 40.5% and 52.3% of wild-type levels, respectively (Figure 6B). These decreases were associated with reduced transcript levels of the fatty acid regulatory genes known to be dependent of PPARα, namely carnitine palmitoyltransferase-1β (CPT-1β), known for controlling fatty acid transfer into mitochondria, and malonyl-CoA decarboxylase-1 (MCD-1) (Figure 6C). Malonyl-CoA is a strong inhibitor of CPT-1β; therefore, reduced decarboxylation by MCD-1 in raptor-cKO mice may, by causing accumulation of malonyl-CoA, contribute to reduced fatty acid oxidation. However, in our study, gene expression of the enzyme responsible for synthesis of malonyl-CoA, acetyl-CoA carboxylase, was decreased concomitantly (Figure 6C), which may attenuate the increase of malonyl-CoA in the myocardium. Finally, gene expression of succinyl-CoA-3-oxoacid CoA transferase, a regulator of ketone body metabolism, was reduced (Figure 6C).

Changes in fatty acid oxidation were associated with changes in glucose transporters. Figure 6D shows that raptor deficiency by itself reduced GLUT4 mRNA levels to 23% of those in wild-type sham mice, an effect far more drastic than that observed after TAC. In contrast, transcripts of GLUT1 were 2.2-fold higher in raptor-cKO than in wild-type mice.
and those of GAPDH, an enzyme of glycolysis, were also increased, consistent with enhanced carbohydrate metabolism. Taken together, our results suggest that mTORC1 is involved in the regulation of metabolic substrate use in the heart.

Raptor Knockout Mice Show Abnormal Mitochondria, Increased Apoptosis, and Increased Autophagy

Besides modulating fatty acid oxidation, PGC1α is known to regulate mitochondrial biogenesis. After TAC, raptor deletion resulted in significantly decreased mitochondrial DNA normalized for genomic NADH (P<0.05; Figure 7A). Moreover, ultrastructural analysis revealed swollen mitochondria with irregular cristae as a feature of raptor-cKO mice (Figure 7B). To identify further mechanisms that potentially contributed to the observed phenotype, we assessed apoptosis by immunohistochemistry (Figure 7C). Cleaved caspase-3 appeared to be increased after raptor deletion, an effect that we confirmed quantitatively by immunoblotting (Figure 7D) and that indicated the activation of apoptotic pathways. mTOR is known to regulate autophagy, a protective mechanism that cells activate in case of nutrient deficiency or other stress. Figure 7E shows that LC3BII was increased after raptor deletion along with ULK1, supporting that autophagy was enhanced. We conclude that changes in mitochondria, apoptosis, and autophagy are part of the cascade of events that precede the development of heart failure after mTORC1 inactivation.

Discussion

In this study, we analyzed the function of mTORC1 in the adult mouse heart by conditionally deleting raptor from cardiomyocytes. Raptor-cKO mice had normal cardiac architecture, function, and metabolism at 2 weeks after gene ablation. Function was maintained for up to 3 weeks, but at
this time point, significant changes in metabolic gene expression, along with a strong induction of ANP, BNP, and β-MHC, indicative of a stress response, were measured. Cardiac function began to deteriorate afterward and developed all features typical of severe dilated cardiomyopathy at 4 weeks after raptor ablation. Pressure overload applied to raptor-cKO mice resulted immediately in severe cardiac dilation with strongly reduced ejection fractions; a prior phase of adaptive cardiomyocyte hypertrophy was missing. With these experiments, we established that mTORC1 is a critical mediator of adaptive ventricular growth in conditions of pressure overload but, importantly, that it also is an essential component for cardiac homeostasis under physiological conditions. Our study provides evidence for a causal relationship between depressed mTORC1 activity and cardiac dysfunction.

Metabolic stress may have been one of the early triggers preceding the dysfunction that developed after 3 weeks and culminated in heart failure in our raptor-cKO mice. This notion is supported by recent studies in which mTORC1 was shown to control mitochondrial gene expression and oxygen consumption via transcriptional mechanisms that involve direct interactions between mTOR and raptor as binding partners of YY1 and PGC1α.29,30 Consistently, PGC1α expression was reduced and mitochondrial content and structure were negatively affected in the raptor-cKO mice, in line with our previous report on skeletal muscle.20 Interestingly, expression of PPARα and ERRα, PGC1α-binding partners that regulate transcription of fatty acid oxidation genes in the heart, was also decreased. The effects were confirmed because CPT-1β and MCD-1 were reduced concomitantly. Furthermore, there was a shift in mRNA expression from GLUT4 to GLUT1, and our working heart experiments indeed showed a switch from fatty acid toward glucose oxidation, typically observed after myocardial infarct and in heart failure.31,32 Along with these metabolic changes, we found an isoform change toward β-MHC, reminiscent of the switch from fast- to slow-twitch muscle described after raptor ablation in skeletal muscle.20 Because β-MHC generates force in an energetically more economic manner than the α isoform, it may represent a compensatory energy-preserving effort after raptor ablation. Taken together, our data demonstrate that raptor deletion changes mitochondria and metabolic gene expression at a time point when cardiac function and geometry are normal and that this is followed by increased carbohydrate metabolism and loss of cardiac function. Further studies are required to elucidate exactly how deletion of raptor induces this shift in the metabolic gene program.

Our study also provides support for a role of mTORC1 in regulating cardiac apoptosis and autophagy because cleaved caspase-3 and LC3B II were increased after raptor ablation. This is in line with what is known for mTORC1 in other cell types in which, for example, the regulation of autophagy involves direct interactions between raptor and ULK1.33 Our results provide additional support for the conclusions of a recent study by Zhang et al14 that were based on the combined inactivation of mTORC1 and mTORC2 after mTOR ablation.

The hearts of sham-operated raptor-cKO mice did not decrease in weight, suggesting either that 4E-BP1- and S6-mediated reductions in protein translation were not sufficient to yield an atrophic response during this time period or alternate pathways of protein synthesis were activated or that degradation via mTORC1-independent pathways was diminished. In support of attenuated degradation, we observed reduced muscle atrophy F-box and MuRF1 mRNA levels as a possible consequence of the hyperphosphorylated Akt in raptor-cKO hearts. Akt hyperphosphorylation has previously been explained by lacking negative feedback through S6K1, a feedback loop that normally causes IRS-1 degradation, thereby controlling Akt phosphorylation.20,21,35 Our present
observation of hyperphosphorylated Akt supports the existence of a similar feedback loop in the heart.

The consequences of blunted protein synthesis were more pronounced after TAC and likely became an important reason for precipitated functional deterioration. Accelerated protein synthesis is required for the adaptive hypertrophic response that preserves cardiac function. We consistently found an increase in total and phosphorylated levels of multiple mediators of protein synthesis in wild-type mice, and their blockade in the raptor-cKO mice explains why ventricular weight and cardiomyocyte cross-sectional area did not increase. The resulting high wall stress likely triggered neurohormonal and inflammatory responses. Moreover, the induction of β-MHC already observed under baseline conditions became significantly more pronounced after TAC and probably contributed to cardiac dysfunction, as reported previously. Finally, MuRF3 was induced in TAC-operated raptor-cKO mice, which, consistent with its role in degrading β-MHC, provides another mechanism for the rapid wall thinning and heart failure.

Consistent with our findings after mTORC1 inactivation, rapamycin treatment resulted in reduced hypertrophic responses to hemodynamic stress. This discrepancy might be explained by the dosing and timing of the rapamycin treatment. Notably, rapamycin fully inactivated S6K1; nevertheless, suppression of the growth response to overload was incomplete. This suggests that rapamycin-resistant effects of mTORC1, recently reported for other systems, may have contributed to incomplete inhibition of hypertrophy and maintenance of cardiac function. In addition, a major difference with our model is that rapamycin affects all compartments of the heart, including fibroblasts, microvasculature, and inflammatory cells. Although our study demonstrates the essential function of mTORC1 in cardiomyocytes in vivo, extensive further studies are required to dissect its relative importance in other cell types of the heart.

Conclusion

Our study demonstrates that mTORC1 activity in cardiomyocytes is critical for the preservation of cardiac function in response to pressure overload. Importantly, we show that mTORC1 is also essential under normal workload conditions. Thus, cardiac raptor deficiency caused severe heart failure with high mortality within 6 weeks after gene ablation in...
mice. Our study underlines that monitoring of cardiac function in clinical studies with mTOR inhibitors is important.

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Disclosures
None.

References


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**CLINICAL PERSPECTIVE**

Mammalian target of rapamycin (mTOR) is an evolutionary conserved kinase that regulates cell growth. Rapamycin is clinically used as an immunosuppressant and as potent antiproliferative agents; several rapalogs are in trials for cancer therapy. Rapamycin inhibits, at least in part, the activities of mTOR in mTOR complex (mTORC) 1, 1 of the 2 distinct multiprotein complexes in which mTOR is found. The recently developed active-site mTOR inhibitors inhibit mTORC1 and mTORC2. In the present study, we established that mTORC1 is essential for normal cardiac function and that its activity becomes even more important with increased cardiac work. Thus, deletion of *raptor*, an essential component of mTORC1, from cardiomyocytes in adult mice caused dilated cardiomyopathy within 6 weeks. Moreover, increased load by dynamic exercise or aortic constriction accelerated progression into heart failure without a prior phase of adaptive hypertrophy. This strengthens the perception that appropriate growth responses with intact signaling via the insulin/insulin-like growth factor/Akt/mTOR pathway are important when the heart has to generate more work, which applies to various clinical conditions such as hypertension, postinfarct remodeling, and valve disease. Notably, this signaling pathway is affected during anticancer therapy and therefore may be involved in the development of cardiomyopathy in these patients. Besides slowing of protein synthesis, we show that cardiac mTORC1 inactivation affects mitochondria, substrate oxidation, autophagy, and apoptosis. These mechanistic insights may lead to the development of novel approaches to prevent cardiac dysfunction. Our study also underlines that monitoring of cardiac performance in clinical trials with active-site mTOR inhibitors is important.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Generation of Inducible, Cardiac-Specific Raptor Knockout Mice

Mice homozygous for loxP-flanked raptor exon 6\textsuperscript{1,2} were crossed with mice expressing Cre recombinase under the control of the cardiomyocyte-specific α-myosin heavy chain (MHC) promoter in a tamoxifen-inducible manner.\textsuperscript{3} Resulting heterozygous floxed raptor mice positive for the α-MHC-MerCreMer transgene (α-MHC-MerCreMer/raptor\textsuperscript{fl/+}) were further mated with homozygous floxed raptor (raptor\textsuperscript{fl/fl}) mice to obtain mice positive for α-MHC-MerCreMer and carrying two floxed raptor alleles (α-MHC-MerCreMer/raptor\textsuperscript{fl/fl}). Mice analyzed in this study were backcrossed to C57BL/6J for 6-8 generations. PCR genotyping of floxed raptor mice was performed using the forward primer: 5’-ATG GTA GCA GGC ACA CTC TTC ATG-3’ and reverse primer: 5’-GCT AAA CAT TCA GTC CCT AAT C-3’, resulting in an amplicon of 228 bp for floxed raptor and of 141 bp in case of wild-type allele. Genotyping of mice for the presence of Cre recombinase was performed using the forward primer 5’-GTT CGC AAG AAC CTG ATG GCA A-3’ and the reverse primer 5’-CTA GAG CCT GTT TTG CAC GTT C-3’ yielding a product of 340 bp for the recombined allele and no product for the wild-type.

Experimental models

For voluntary exercise, mice were individually housed in cages equipped with a running wheel.\textsuperscript{1} A sensor was attached to the wheel and connected to a computer for continuous monitoring of running activity. Transverse aortic constriction was performed and cardiac function determined using the Vevo 770 Ultrasonograph (VisualSonics) according to published procedures.\textsuperscript{1,4,5} Both models were started at two weeks after the last tamoxifen injection.
Wheat Germ Agglutinin Staining and Immunohistochemistry

Hearts were arrested with ice cold 0.9% NaCl and frozen in OCT (Medite, Nunningen, Switzerland) using isopentane cooled in liquid nitrogen. Cryosections were fixed for 20 min at RT with 4% paraformaldehyde and washed with PBS-glycine (100 mmol/l) for 10 min. Sections were permeabilised with 0.1% Triton X-100 for 20 min and incubated with FITC-labeled wheat germ agglutinin (4 µg/ml) for 90 min at RT. Cross sectional areas of at least hundred cardiomyocytes in three independent sections of 3-4 mice per group were measured. For immunohistochemistry, sections were incubated with antibodies to β-MHC (Abcam) and cleaved caspase-3 (Becton, Dickinson and Company), followed by Cy3- (Jackson Immunoresearch) or Alexa555- (Molecular Probes) labeled secondary antibodies. Nuclei were stained with DAPI (Sigma, 1 µg/ml).

Protein Extraction and Western Blot Analysis

Tissue for protein analysis was flash-frozen in liquid nitrogen and stored at −80°C. Total protein was extracted using a Polytron homogenizer and RIPA buffer (50 mmol/l Tris-HCl, pH=7.4, 150 mmol/l NaCl, 1% NP40, 0.25% Na deoxycholate, 5 mmol/l EDTA, 10 µmol/l leupeptin, 1 mg/ml benzamidine, 100 U/ml bacitracin, 0.1 TIU/mL aprotinin, 1 mg/ml TAME, 1 mg/ml BAEE, 10 mmol/l Na-pyrophosphate, 10 mmol/l glycerophosphate 0.5 % phosphatase inhibitor cocktail 1 and 2 (Sigma), 2 mmol/l Pefabloc plus and "Mini-Complete" protease inhibitor cocktail (Roche Diagnostics)). Equal amounts of protein were separated on SDS-PAGE and after transfer to PVDF membrane, incubated overnight with primary antibody. The unbound primary antibody was removed by 3-4 consecutive washings, the membrane incubated with IRDye labeled secondary antibody for 1 h, and the signal detected and quantitated using Odyssey imaging software (LI-COR Biosciences, Lincoln, Nevada, USA).

Polyclonal rabbit antibodies against phospho-Akt (Ser473), phospho-Akt (Thr308), Akt total, GSK3β (Ser9), phospho-4E-BP1 (Thr70), 4E-BP1 total, phospho-S6 kinase (Ser240/244), p70 S6 kinase, mTOR and monoclonal antibody against raptor were all from Cell Signaling Technology (Danvers, MA). The GAPDH mouse monoclonal antibody and ANP rabbit polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).
RNA Preparation and Quantitative RT-PCR

Total RNA was extracted from frozen hearts using Tri Reagent (Sigma) and treated with DNAse I (Ambion, Austin, TX). Concentration, purity and quality of the RNA were assessed by spectrophotometry and agarose gel electrophoresis. cDNA was prepared from these total RNA extracts using the high capacity DNA reverse transcription kit (Applied Biosystems). The product was diluted 1:100 and 5 µl were amplified on a 7500 fast real-time PCR system (Applied Biosystems), with 1x ITaQ SYBR Green Supermix Kit (Bio-Rad, Reinach, Switzerland) and 300 nmol/l for forward and reverse primers in a total volume of 20 µl. The mRNA level was based on the critical threshold (Ct) value. The primers used for the real time PCR were designed with the software Primers Express (Applied Biosystems, Foster City, CA) and synthesized by Microsynth (Balgach, Switzerland). Primer sequences for quantitative real-time PCR are provided in the following table:

Table: Primers used in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-CGG CCG CAT CTT CTT GTG-3'</td>
<td>5'-CAC CGA CCT TCA CCA TTT TGT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CAG CTT CTT TGC AGC TCC TT-3'</td>
<td>5'-GCA GCG ATA TCG TCA TCC A-3'</td>
</tr>
<tr>
<td>ANP</td>
<td>5'-TGG GAC CCC TCC GAT AGA TC-3'</td>
<td>5'-TCG TGA TAG ATG AAG GCA GGA A-3'</td>
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<tr>
<td>α-MHC</td>
<td>5'-CTA CGC GCC CTCG TAT AGA TC-3'</td>
<td>5'-GCC ACT TGT AGG GGT TGG TTG A-3'</td>
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<tr>
<td>β-MHC</td>
<td>5'-TTG AGA ATC CAA GCC TCA GC-3'</td>
<td>5'-CTT CTC AGA TCT CCG CAG GA-3'</td>
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<tr>
<td>α-sk.actin</td>
<td>5'-CAG CTC TGG CTC CCA GCA CC-3'</td>
<td>5'-AAT GGC TGG CTT TAA TGC TTC A-3'</td>
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<tr>
<td>GLUT1</td>
<td>5'-GGG CAT GTG CCT CCA GTA TGT-3'</td>
<td>5'-ACG AGG AGC ACC GTG AAG AT-3'</td>
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<td>GLUT4</td>
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<td>5'-CCG ACT CGA AGA TGC TGG TTG AA-3'</td>
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<tr>
<td>PGC1α</td>
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<td>5'-TCT CTC AGA CTA CAA GGA AGA C-3'</td>
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<td>MAFbx</td>
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<td>5'-GGC TGC TGA ACA GAT TCT CC-3'</td>
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<td>5'-GCC CGC CAC CAG CAT-3'</td>
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<tr>
<td>MCD1</td>
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<td>5'-TCG GAG GGC ACT CCT TCA-3'</td>
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<tr>
<td>CPT1β</td>
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<td>5'-GTC TCA TCG TCA GGG TTG TAG CT-3'</td>
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<td>ACC2</td>
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<td>5'-GAA ATC TCT GTG CAG GTG TGT TT-3'</td>
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<tr>
<td>PPARα</td>
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<tr>
<td>SCOT</td>
<td>5'-AAG CCA TCA CGG GAG ATT TT-3'</td>
<td>5'-CCA CGG TAG TGC TGT CAG C-3'</td>
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</table>
Glucose and Palmitate Oxidation and Cardiac Function During Ex Vivo Heart Perfusion

Cardiac function and substrate oxidation were measured in isolated perfused hearts essentially as described. Briefly, the hearts were perfused with a modified Krebs–Henseleit bicarbonate buffer supplemented with 0.5 mmol/l palmitate, 5 mmol/l glucose and 100 µU/ml insulin. The preload pressure was 7.5 mm Hg, while hearts were ejecting against an afterload of 50 mm Hg. The amount of $^3$H$_2$O released from [9,10-$^3$H] palmitate was measured to determine fatty acid oxidation and the amount of $^{14}$CO$_2$ released from [U-$^{14}$C] glucose to determine glucose oxidation. Metabolic rates were normalized for cardiac mass. Functional data were obtained since a 2-Fr micromanometer-conductance catheter (Millar Instruments, Houston, TX) was inserted in the left ventricle through the apex. Developed pressure, cardiac output, cardiac power and myocardial oxygen consumption (MVO$_2$) were analyzed as described.

Transmission Electron Microscopy (TEM)

Mouse heart was perfused with 2.5% glutaraldehyde in 0.1 mol/l sodium phosphate (pH7.4), for 10-15 min. After isolation of heart, cardiac muscles were post-fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 mol/l sodium phosphate. The next day, heart was cut into small pieces of around 2 mm$^3$. After treatment with 1% OsO$_4$ in 0.1 mol/l cacodylate buffer (pH 7.2), muscle pieces were rinsed in 1% Na$_2$SO$_4$ in 0.1 mol/l cacodylate buffer and embedded in Epon. Sections (60-nm thick) were cut on a microtome (Ultracut E; Leica) and post-stained with uranyl acetate and lead citrate. The specimens were examined with an electron microscope (Philips EM400) at an accelerating voltage of 80 kV.

Measurement of Mitochondrial Content

DNA was purified from frozen heart tissue using a routine phenol/chloroform protocol after proteinase K digestion. The amount of mtDNA and genomic DNA was measured by quantitative PCR using the following primers. Mitochondrial DNA, D-loop non-coding region: forward GGTTCTTACTTCAGGGCCATCA, reverse GATTAGACCCGTTACCATCAGAGAT; Genomic DNA, NADH dehydrogenase flavoprotein 1 ($Nduf1$): forward CTTCCCCACTGGCCTCAAG, reverse: CCAAAAACCAGTGATCCAGC.
## Supplemental Table

Table SI: Physiological and echocardiography parameters of wild-type and raptor-cKO mice prior to surgery.

<table>
<thead>
<tr>
<th>Echocardiography</th>
<th>Wild-type</th>
<th>Raptor cardiac knockout</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre-sham (n = 7)</td>
<td>Pre-TAC (n = 11)</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>433±11</td>
<td>461±15</td>
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<tr>
<td></td>
<td>453±11</td>
<td>461±13</td>
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<tr>
<td>Septum thickness (mm)</td>
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<tr>
<td>Diastole</td>
<td>0.70±0.02</td>
<td>0.73±0.01</td>
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<td>0.72±0.01</td>
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<tr>
<td>Systole</td>
<td>0.88±0.02</td>
<td>0.92±0.02</td>
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<tr>
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<td>0.89±0.02</td>
<td>0.89±0.02</td>
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<tr>
<td>Left ventricular free wall thickness (mm)</td>
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<td></td>
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<tr>
<td>Diastole</td>
<td>0.70±0.01</td>
<td>0.73±0.01</td>
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<tr>
<td>Systole</td>
<td>0.88±0.02</td>
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<tr>
<td></td>
<td>0.88±0.02</td>
<td>0.89±0.01</td>
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<tr>
<td>Left ventricular diameter (mm)</td>
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<tr>
<td>Diastole</td>
<td>4.06±0.06</td>
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<td>4.21±0.10</td>
<td>4.28±0.06</td>
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<tr>
<td>Systole</td>
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<td>3.35±0.13</td>
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<tr>
<td>Ejection fraction (%)</td>
<td>46.3±1.4</td>
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<td>41.9±3.3</td>
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<td>Fraction of shortening (%)</td>
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<td>20.5±1.9</td>
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<td>Body weight (g)</td>
<td>25.9±0.6</td>
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<td>26.7±0.6</td>
<td>27.8±0.6</td>
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</table>
Supplemental References


