Rheb is a Critical Regulator of Autophagy During Myocardial Ischemia
Pathophysiological Implications in Obesity and Metabolic Syndrome

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Background—Rheb is a GTP-binding protein that promotes cell survival and mediates the cellular response to energy deprivation (ED). The role of Rheb in the regulation of cell survival during ED has not been investigated in the heart.

Methods and Results—Rheb is inactivated during cardiomyocyte (CM) glucose deprivation (GD) in vitro, and during acute myocardial ischemia in vivo. Rheb inhibition causes mTORC1 inhibition, because forced activation of Rheb, through Rheb overexpression in vitro and through inducible cardiac-specific Rheb overexpression in vivo, restored mTORC1 activity. Restoration of mTORC1 activity reduced CM survival during GD and increased infarct size after ischemia, both of which were accompanied by inhibition of autophagy, whereas Rheb knockdown increased autophagy and CM survival. Rheb inhibits autophagy mostly through Atg7 depletion. Restoration of autophagy, through Atg7 reexpression and inhibition of mTORC1, increased cellular ATP content and reduced endoplasmic reticulum stress, thereby reducing CM death induced by Rheb activation. Mice with high-fat diet–induced obesity and metabolic syndrome (HFD mice) exhibited deregulated cardiac activation of Rheb and mTORC1, particularly during ischemia. HFD mice presented inhibition of cardiac autophagy and displayed increased ischemic injury. Pharmacological and genetic inhibition of mTORC1 restored autophagy and abrogated the increase in infarct size observed in HFD mice, but they failed to protect HFD mice in the presence of genetic disruption of autophagy.

Conclusions—Inactivation of Rheb protects CMs during ED through activation of autophagy. Rheb and mTORC1 may represent therapeutic targets to reduce myocardial damage during ischemia, particularly in obese patients. (Circulation. 2012;125:1134-1146.)

Key Words: apoptosis ■ ischemia ■ obesity ■ signal transduction

Heart failure is viewed as one of the major healthcare problems worldwide, with acute myocardial infarction (MI) as the most common predisposing cause. It is fundamental to clarify the mechanisms regulating cardiomyocyte (CM) death and survival during ischemic injury to find new therapies to reduce the amount of myocardial loss after a sudden coronary occlusion.

Clinical Perspective on p 1146
Ras homolog enriched in brain (Rheb) is a small GTP-binding protein that has been shown to regulate the cellular stress response, both in lower organisms and in mammalian cell lines. In particular, Rheb appears to be a critical sensor of energy stress, being inactivated under this condition. Inhibition of Rheb during cellular stress promotes the upregulation of adaptive mechanisms, such as cell cycle arrest and growth inhibition, which may save energy, favor DNA repair, and thus be protective. On the contrary, Rheb is hyperactivated in cancer cells, where it promotes stress resistance and survival, and Rheb activation was found to directly inhibit apoptotic pathways induced by amino acid deprivation and genotoxic stress. Therefore, it is not clear whether Rheb activity is protective or detrimental during cellular stress. Remarkably, the role of Rheb in response to acute energy deprivation, and in regulation of cell death and survival, has never been investigated in the heart.

Rheb activity is regulated by upstream kinases, such as Akt, AMP-activated protein kinase (AMPK), and glycogen...
synthase kinase-3α, which control Rheb through direct modulation of the heterodimer composed of the tuberous sclerosis complex proteins 1 (TSC1) and 2 (TSC2). The TSC1/TSC2 complex inhibits Rheb by exerting a strong GTPase activity toward it.² ⁷ Rheb directly binds and selectively activates the multiprotein complex 1 of mammalian target of rapamycin (mTORC1), which in turn mediates many cellular functions, such as protein translation.⁸ mTORC1 is also inhibited in response to energy stress, and its inactivation reduces protein synthesis and upregulates autophagy.⁹ However, mTORC1 activation also promotes cell survival and inhibits apoptosis in several stress conditions, and, therefore, whether mTORC1 inhibition is detrimental or protective during cellular stress is stimulus dependent.⁵ ⁸

The role of mTORC1 in mediating survival and death of CMs has only been investigated in models of chronic cardiac remodeling, with discordant results.⁹ ¹⁻⁻¹¹ Important, the effect of direct and selective mTORC1 versus mTORC2 modulation during CM acute energy deprivation, such as myocardial ischemia, remains to be elucidated. It is also unclear how mTORC1 is modulated during CM energy deprivation and whether Rheb, an immediate upstream regulator of mTORC1, is critically involved in such regulation in CMs.

In our study, we investigated the role of Rheb in the regulation of cell death and survival during CM starvation and ischemia, and the underlying molecular mechanisms. In particular, we studied whether a direct and selective modulation of mTORC1 induced by Rheb is involved in the effects exerted by Rheb on CM survival during energy stress. Recent reports have shown that obesity and metabolic syndrome, which are characterized by an increased risk of cardiovascular mortality¹² and increased myocardial susceptibility to ischemic injury,¹³⁻⁻¹⁵ are associated with a hyperactivation of tissue mTORC1.¹⁷ ¹⁸ Therefore, we also evaluated whether cardiac mTORC1 is activated in obesity and metabolic syndrome, whether Rheb is involved in such phenomena, and whether a deregulated activation of Rheb and mTORC1 may be responsible for the increased susceptibility to ischemia associated with these conditions.

Methods

Experimental Procedures

Experimental procedures and animal models are described in the expanded Methods section in the online-only Data Supplement. Experimental procedures, heterozygous GFP-LC3 transgenic mice, beclin-1 knockout mice, and conditional mTOR knockout mice have also been described elsewhere.⁹ ¹⁹ ²⁰ All experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

Statistics

Data are expressed as mean±SEM. When specified in the figure legends, presentation of bar charts was standardized by control mean×100, so that the presented bars represent the mean percentage of variation±SEM, with respect to the control mean. The difference in means between 2 groups was evaluated using the t test when sample size was appropriate and the population was normally distributed; otherwise, the Mann-Whitney U test was adopted. When differences among 3 or more groups were evaluated, the one-way analysis of variance or the Kruskal-Wallis test was used. The post hoc comparisons were performed by use of the Bonferroni post hoc test or the Mann-Whitney U test with Bonferroni correction. The shown statistical significance of differences between groups was always calculated by post hoc comparisons when multiple groups were compared. Statistical analyses were performed with the use of SPSS 15.0 (SPSS Inc, Chicago, IL) and GraphPad-Prism 5.00 (GraphPad-Software, San Diego, CA). Probability values of <0.05 were considered statistically significant.

Results

Rheb Mediates mTORC1 Inhibition During Starvation in CMs

To investigate whether Rheb acts as a sensor of energy deprivation in CMs, neonatal rat ventricular CMs were subjected to glucose deprivation (GD). GTP binding of Rheb was decreased significantly in response to GD (Figure 1A), indicating that Rheb is inactivated by GD. During GD, phosphorylation of p70S6K and 4E-BP1 was progressively reduced, indicating that mTORC1 was inhibited (Figure 1B). Knockdown of Rheb, with adenovirus harboring shRNA-Rheb, inhibited phosphorylation of p70S6K at baseline, suggesting that inactivation of Rheb is sufficient to inactivate mTORC1 (online-only Data Supplement Figure IA). Transduction of CMs with adenovirus harboring wild-type Rheb abolished the GD-induced decreases in phosphorylation of p70S6K and 4E-BP1 (Figure 1C through 1E and online-only Data Supplement Figure IB), suggesting that Rheb inactivation is required for GD-induced suppression of mTORC1. In addition, Rheb physically interacts with mTOR both at baseline and during GD (online-only Data Supplement Figure IC), thus indicating that Rheb directly regulates mTORC1 in CMs.

Rheb is negatively regulated by the GTPase-activating protein activity of the TSC1/TSC2 complex.⁷ Downregulation of TSC2, with adenovirus harboring shRNA-TSC2 (online-only Data Supplement Figure ID), induced phosphorylation of p70S6K, suggesting that endogenous TSC2 negatively regulates mTORC1 in CMs (online-only Data Supplement Figure IE and IF). Activation of mTORC1 by downregulation of TSC2 was abolished in the presence of Rheb knockdown, suggesting that TSC2 regulates mTORC1 through Rheb (online-only Data Supplement Figure IE and IF). On the other hand, as indicated by the phosphorylation status of Akt, the activity of mTORC2, another branch of the mTOR pathway, was unaffected by GD, and overexpression of Rheb failed to activate Akt (online-only Data Supplement Figure IG and IH). These results suggest that GD inhibits mTORC1, but not mTORC2, by inactivating Rheb.

Activation of Rheb Sensitizes CMs to Cell Death During GD, Whereas Inhibition Is Protective

We then investigated the role of Rheb in regulating CM survival during GD. Although Rheb is cell protective in other cell types, CMs in which the activity of mTORC1 was normalized with overexpressed Rheb displayed decreased survival after 10 and 18 hours of GD in comparison with control virus–treated CMs (Figure 2A). CMs overexpressed with Rheb displayed significantly more apoptosis and necrosis, as assessed by TdT-mediated dUTP nick-end labeling (TUNEL) assays and propidium iodide staining, respectively.
TSC2 knockdown also decreased survival and increased apoptosis of CMs in response to GD (Figure 2D and online-only Data Supplement Figure IIB). Conversely, downregulation of endogenous Rheb increased the survival of CMs during GD and rescued the decrease in cell survival in the presence of TSC2 knockdown during GD (Figure 2E). Furthermore, selective inhibition of mTORC1, through Raptor downregulation, with adenovirus harboring shRNA-Raptor, significantly increased CM survival during GD in Rheb-overexpressing CMs. In contrast, selective mTORC2 inhibition, through Rictor depletion, did not increase survival in Rheb-overexpressing CMs during GD (Figure 2F). Collectively, these data suggest that Rheb negatively regulates CM survival during GD through mTORC1 activation. Thus, inactivation of endogenous Rheb during GD is an adaptive mechanism that promotes survival of CMs.

**Rheb Regulates CM Autophagy**

We investigated the molecular mechanism through which inactivation of Rheb protects CMs during GD. Because Rheb inactivation causes mTORC1 inhibition during GD, and because mTOR is a negative regulator of autophagy, we hypothesized that downregulation of Rheb during GD is required for stimulation of autophagy, which may be protective in this context. As shown previously, GD increased LC3-II and decreased p62, a protein degraded by autophagy, suggesting that GD activates autophagy in CMs. However, in Rheb-overexpressing CMs, LC3-II expression was lower and expression of p62 was greater, both at baseline and during GD (Figure 3A through 3C). The number of GFP-LC3 dots, an indicator of autophagosome accumulation, during GD was significantly smaller in Rheb-overexpressing CMs than in control CMs (Figure 3D and 3E). Rheb overexpression induced significant downregulation of autophagy genes, including beclin-1, ulk-1, atg4, and atg7 (Figure 3F). Conversely, downregulation of endogenous Rheb significantly increased autophagy at baseline and during GD (online-only Data Supplement Figure III). These results suggest that endogenous Rheb negatively regulates autophagy and that inactivation of Rheb is necessary and sufficient for stimulation of autophagy in CMs during GD.
We then asked if autophagy mediates the cell-protective effect of Rheb inactivation. The protective effect of Rheb downregulation during GD was completely abrogated when Beclin-1 was downregulated with adenovirus harboring shRNA-beclin1 (Figure 3G). These results suggest that autophagy plays an important role in mediating the protective effect of Rheb inactivation during GD. Conversely, to restore autophagy during GD in Rheb-overexpressing CMs, we expressed Atg7 with adenovirus transduction (online-only Data Supplement Figure IVA). We took this approach because Atg7 is a crucial protein for autophagosome formation, because Atg7 is markedly downregulated in Rheb-overexpressing CMs, and because overexpression of Atg7 is sufficient to reinduce autophagy when autophagy is inhibited.\textsuperscript{21,22} Atg7 overexpression significantly restored autophagy in Rheb-overexpressing CMs during GD (online-only Data Supplement Figure IVB through IVD). We also used trehalose, which induces autophagy without affecting the mTORC1 pathway.\textsuperscript{23} Trehalose restored Atg7 and Beclin-1 expression in Rheb-overexpressing CMs without affecting the mTORC1 pathway, thereby restoring autophagy during GD (online-only Data Supplement Figure IVE and IVF). Importantly, both Atg7 expression and trehalose pretreatment significantly increased the survival of Rheb-overexpressing CMs during GD (Figure 3H). Collectively, these results suggest that Rheb regulates the survival and death of CMs during GD through regulation of autophagy in vitro.

Rheb Overexpression Increases Energy Stress and Endoplasmic Reticulum Stress During GD

Important consequences of autophagy include restoration of ATP contents and protein quality control. Rheb overexpression during GD significantly enhanced ATP depletion, whereas Rheb disruption significantly increased ATP content (Figure 4A). Overexpression of Rheb increased GRP78, phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase and upregulation of Ccaat-enhancer-binding protein homologous protein (CHOP), and Caspase-12 (fragment), markers of endoplasmic reticulum (ER) stress, in CMs during GD (Figure 4B and 4C). Conversely, upregulation of the ER stress markers during GD was significantly attenuated in CMs in which Rheb was knocked down (Figure 4B and 4C). Restoration of autophagy in Rheb-overexpressing CMs,
through Atg7 overexpression, significantly attenuated ATP depletion (Figure 4D) and ER stress during GD, indicating that autophagy inhibition is responsible for these derangements (Figure 4E and 4F).

Inhibition of Rheb Is Protective During Prolonged Myocardial Ischemia

To investigate the role of Rheb in regulating CM survival and death in response to energy deprivation in vivo, we used a mouse model of prolonged ischemia, in which the left descending coronary artery was ligated for 3 hours. During ischemia, the GTP-bound form of Rheb was significantly decreased, whereas the total expression of Rheb was not altered, suggesting that Rheb is inactivated by prolonged ischemia in vivo (Figure 5A and 5B). The activity of mTORC1, as evaluated with p70S6K phosphorylation, was also decreased during ischemia (Figure 5C and 5D).

To evaluate the significance of Rheb inhibition during ischemia in vivo, we generated transgenic mice with cardiac-specific overexpression of Rheb (Tg-Rheb) with use of a Tet-off system. In these mice, expression of the Rheb transgene in the heart was induced in the absence of doxycycline. Doxycycline was administered to the mice during the gestational period and for the first 3 to 4 weeks of life to avoid
the effect of transgene expression during cardiac development in Tg-Rheb (Figure 5E and online-only Data Supplement Figure VA and VB). Doxycycline was terminated 6 to 8 weeks before the experiment to allow full transgene expression and eliminate possible actions of doxycycline on cell death/survival. In this protocol, expression of Rheb was 2.3-fold greater in Tg-Rheb than in control littermates (Rheb+/	TA− mice). Rheb exhibited diffuse cytoplasmic distribution in CMs of both control mice and Tg-Rheb (online-only Data Supplement Figure VC and VD). Tg-Rheb presented a normal cardiac phenotype at 3 months of age (online-only Data Supplement Table I). In Tg-Rheb mice, mTORC1 activity was significantly increased, both at baseline and during prolonged ischemia, in comparison with control mice (Figure 5C and 5D), suggesting that Rheb inactivation is required for mTORC1 inhibition during prolonged ischemia. After 3 hours of ischemia, Tg-Rheb mice exhibited a significantly greater MI size than control mice (Figure 5F through 5H). The extent of CM apoptosis and necrosis after prolonged ischemia was also greater in Tg-Rheb than in controls, as evaluated with TUNEL and Hairpin-2 staining, respectively (online-only Data Supplement Figure VI). Tg-Rheb presented increased ischemic injury even after a brief period of ischemia (30 minutes), as evaluated with Hairpin-2 staining. Tg-Rheb also exhibited significantly enhanced myocardial damage even after a longer coronary occlusion (6 hours; online-only Data Supplement Figure VII).

There was less induction of autophagy in Tg-Rheb than in control mice at baseline and during ischemia, as indicated by reduced LC3-II and increased p62 accumulation (online-only Data Supplement Figure VIII A through VIII C). Expression of p62 did not differ at the mRNA level between controls and Tg-Rheb, indicating that increased p62 accumulation was due to reduced degradation (mRNA expression in Tg-Rheb 0.96-fold versus controls, \( P = \text{NS} \)). The level of myocardial ATP in the ischemic area after ischemia was significantly lower in Tg-Rheb than in controls (online-only Data Supplement Figure VIII D). The level of CHOP, an indicator of ER stress, after ischemia was also significantly greater in Tg-Rheb than in control mice (online-only Data Supplement Figure VIII E and
These results indicate that inhibition of endogenous Rheb is protective during prolonged ischemia in vivo. To evaluate whether the deleterious effect of Rheb overexpression during prolonged ischemia is due to the lack of mTORC1 inactivation and activation of autophagy, rapamycin, a selective inhibitor of mTORC1 and stimulator of autophagy, was administered to Tg-Rheb and control mice just before the prolonged ischemia. Rapamycin inhibited mTORC1 activity and stimulated autophagy in Tg-Rheb mice after prolonged ischemia (Figure 6A). Rapamycin significantly reduced the size of MI in response to prolonged ischemia in Tg-Rheb mice in comparison with vehicle administration (Figure 6B through 6D). Rapamycin treatment also significantly reduced the size of MI after prolonged ischemia (Figure 6E and 6F). These results suggest that Rheb promotes myocardial injury during prolonged ischemia by stimulating mTORC1, inhibiting autophagy and stimulating ER stress.

High-Fat Diet–Induced Obesity Is Associated With Deregulation of Rheb and Increased Myocardial Susceptibility to Prolonged Ischemia

Obesity and metabolic syndrome are associated with high cardiovascular mortality and reduced cardiac function after MI. Complications of obesity are associated with deregulated mTORC1 activation and inhibition of autophagy in other organs. We therefore investigated whether obesity is associated with deregulated Rheb activation, which in turn mediates an increased susceptibility to myocardial ischemia.

To induce obesity, C57BL/6J mice were fed with high-fat diet (HFD) mice for 18 to 20 weeks. HFD mice developed obesity and exhibited a significant increase in serum levels of glucose, cholesterol, triglycerides, and nonesterified fatty acid in comparison with mice fed with control diet (CD) mice, suggesting that HFD mice develop metabolic syndrome (online-only Data Supplement Table II). Insulin levels and the HOMA index were significantly elevated in HFD mice, consistent with the notion that these...
mice develop insulin resistance. HFD mice presented increases in mTOR-dependent IRS-1 phosphorylation (serine 636), which is a marker of decreased insulin sensitivity at baseline (online-only Data Supplement Figure IXA). HFD mice showed a significant increase in left ventricular mass and left ventricular wall thickness but preserved left ventricular systolic function (online-only Data Supplement Table III). Both cell size and expression of atrial natriuretic factor, a fetal-type gene, were increased, suggesting that HFD mice develop cardiac hypertrophy (online-only Data Supplement Figure IXB through IXD). After prolonged (3 hours) ischemia, HFD mice exhibited a significantly greater MI size than CD mice (Figure 7A through 7C), which was accompanied by greater numbers of TUNEL-positive and Hairpin-2-positive cells (online-only Data Supplement Figure X), signifying that HFD increases myocardial susceptibility to ischemic injury. HFD mice also presented a greater percentage of hairpin-2–positive cells with respect to control mice after 30 minutes of ischemia (11.5±1.3% versus 4.2±1.0%, P<0.05).

In HFD mice, the activity of mTORC1 was greater at baseline and remained elevated during prolonged ischemia (Figure 7D and online-only Data Supplement Figure XIA). Thus, the suppression of mTORC1 in response to prolonged ischemia observed in CD mice was attenuated in HFD mice. Although the GTP-bound form of Rheb was significantly reduced during prolonged ischemia in CD mice, it was increased at baseline and not significantly diminished during prolonged ischemia in HFD mice (Figure 7E), suggesting that the activity of Rheb and mTORC1 is elevated at baseline and remains greater in HFD mice than in CD mice during prolonged ischemia. Intriguingly, the activity of AMPK, a negative regulator of the Rheb/mTORC1 pathway, was reduced in HFD mice both at baseline and during ischemia, as indicated by a reduction in its phosphorylation status. Conversely, it was activated in CD mice during ischemia (Figure 7D and online-only Data Supplement Figure XIA).

Consistent with activation of the mTORC1 pathway, autophagy in the heart was significantly suppressed in HFD mice both at baseline and during ischemia, as indicated by decreased LC3-II and increased p62 accumulation (Figure 7F, online-only Data Supplement Figure XIB and XIC). p62 mRNA expression was unchanged (0.84-fold versus CD
mice, \( P=\text{NS} \)). Accumulation of autophagosomes, as evaluated by use of GFP-LC3 dots, was significantly less in HFD mice than in CD mice (Figure 7G and 7H). The number of GFP-LC3 dots was significantly reduced in HFD mice during ischemia, also after administration of chloroquine, which inhibits lysosomal enzyme activity. These data indicate reduced autophagosome formation in HFD mice (online-only Data Supplement Figure XID and XIE).

To investigate if deregulated activation of the mTORC1 pathway is responsible for the reduced tolerance to prolonged ischemia of HFD mice, we administered rapamycin to these animals and evaluated its effect on ischemic injury. As we observed with Tg-Rheb, rapamycin treatment increased autophagy (online-only Data Supplement Figure XIF) and significantly reduced the MI size of both HFD mice and CD mice (Figure 8A through 8C). Rapamycin administration failed to reduce ischemic injury in heterozygous beclin-1 knockout mice (beclin-1+/-), in which autophagy cannot be activated, when fed with HFD (Figure 8D and 8E). These results indicate that autophagy reactivation mediates the beneficial effect of mTORC1 inhibition in HFD mice.

**Figure 7.** HFD-induced obesity is associated with greater myocardial injury and deregulated Rheb/mTORC1 activation. A through C, MI/AAR was evaluated in CD and HFD mice after ischemia. Bar=1 mm. D, Phosphorylation statuses of p70\textsuperscript{S6K} and AMPK (Thr 172) were evaluated both at baseline and after 30 minutes of ischemia (densitometric analysis is shown in online-only Data Supplement Figure XIA). E, Myocardial Rheb-bound GTP content was evaluated in CD and HFD mice, both at baseline and after 30 minutes of ischemia. \( n=5 \) for each group. F, Myocardial autophagy in HFD mice was significantly inhibited in comparison with control mice, both at baseline and after 30 minutes (LC3-II levels) or 3 hours of ischemia (p62 levels). Representative immunoblots are presented, and densitometric analysis is reported in online-only Data Supplement Figure XIB and XIC. G and H, Tg-GFP-LC3 mice fed with CD or HFD were subjected to ischemia. Representative heart sections are shown. Bar=50 \( \mu \)m. Arrows indicate autophagosomes (G). The number of autophagosomes per microscopic field in the 2 groups after 30 minutes of ischemia is reported (H). \( n=4 \) each group. HFD indicates high-fat diet; CD, control diet; AAR, area at risk; MI, myocardial infarct.
To further demonstrate that deregulated mTORC1 activation increases the ischemic susceptibility of HFD mice, we subjected mice, with inducible cardiac-specific heterozygous mTOR knockout, that were fed with HFD, to prolonged ischemia. This strategy allowed us to partially inhibit the mTOR pathway and to normalize the increased activation of mTORC1 observed in HFD mice. Tamoxifen was administered to \(\text{mTOR}^{+/\text{-}}\)-myosin heavy chain promoter-MerCreMer-mTOR flox/+ mice (\(\text{mTOR}^{+/\text{-}}\)) for 7 days. Cardiac mTOR protein levels were reduced in \(\text{mTOR}^{+/\text{-}}\) mice 3 weeks after tamoxifen administration (online-only Data Supplement Figure XIIA). Cardiac mTOR deletion reduced mTORC1 activity and increased autophagy in \(\text{mTOR}^{+/\text{-}}\) mice fed with control diet or HFD (online-only Data Supplement Figure XIIIB through XIIID). Remarkably, cardiac mTOR deletion reduced MI size in mice fed with control diet and HFD with respect to controls (Figure 8F through 8H). In summary, cardiac activation of the Rheb-mTORC1 pathway in HFD-induced obesity is detrimental during prolonged ischemia because of the inhibition of autophagy.

Discussion

We have demonstrated that Rheb is inhibited in response to GD and prolonged ischemia, and inhibition of Rheb, in turn, inhibits mTORC1 in CMs. Forced activation of Rheb in such conditions stimulates ATP depletion and ER stress by suppression of autophagy, thereby inducing cell death. Thus, Rheb acts as a sensor of energy stress and as a critical regulator of CM survival in response to energy starvation.

We have shown previously that both GD and ischemia in CMs and in the heart, respectively, induce suppression of mTOR.\(^{19,20}\) It should be noted that mTOR is inhibited by both Rheb-dependent and -independent mechanisms.\(^{19,20}\) For example, several upstream kinases, including AMPK and glycogen synthase kinase-3β, which indirectly regu-
late mTOR, inhibit Rheb through phosphorylation and consequent activation of GTPase-activating protein activity in TSC2. It should be noted, however, that mTOR is also inhibited through Rheb-independent mechanisms, such as Akt-dependent phosphorylation of mTOR and FRAS40, and AMPK-dependent Raptor phosphorylation. Our results indicate that Rheb interacts with mTOR, that Rheb is inhibited during GD and myocardial ischemia, and that its inactivation is required for mTORC1 inhibition. Conversely, neither overexpression of Rheb nor GD affected the activity of mTORC2. We therefore propose that Rheb acts as a central and direct regulator of mTORC1 during energy starvation in CMs.

Our results suggest that forced Rheb activation exacerbates cell death and apoptosis during GD and prolonged ischemia, but it does not affect cell survival at baseline. On the other hand, downregulation of Rheb increased survival of CMs during GD, intimating the involvement of endogenous Rheb in the regulation of survival/death during GD and prolonged ischemia. Previous studies have indicated that Rheb promotes cell survival and inhibits apoptotic cell death in response to stress in several cancer cells. Activation of Rheb in un-stressed conditions also induces hypertrophy without cell death in CMs (not shown). Thus, the function of Rheb in cells appears to be context dependent.

Importantly, we found that downregulation of mTORC1 mediates the protective effects of Rheb inhibition during energy deprivation, because depletion of Raptor but not of Rictor, which are the adaptor proteins of complex 1 and complex 2 of mTOR, respectively, increased survival of Rheb-overexpressing CMs during GD in vitro. In addition, pharmacological and genetic inhibition of mTORC1 reduced the susceptibility to ischemic myocardial damage of Rheb-overexpressing and obese mice, and a protective effect was also observed in control animals in vivo. These results suggest that Rheb is an obvious therapeutic surrogate of mTORC1, to achieve increased CM survival during energy deprivation.

The role of mTOR in regulating the stress response is poorly understood in terminally differentiated cell types such as CMs. In particular, the role of mTOR in the regulation of CM survival has been primarily investigated through indirect means, eg, the use of pharmacological inhibitors, which may have mTOR-independent effects. In addition, the role of mTOR in cardiac stress has been mostly studied in animal models of chronic ventricular remodeling in which mTORC1 is activated, whereas we observed mTORC1 inhibition during CM energy deprivation. Interestingly, mTORC1 activation has been indicated as protective during cardiac mechanical overload. On the other hand, in our study, we demonstrated that selective and direct mTORC1 activation is detrimental during acute cardiac energy deprivation, whereas both pharmacological and genetic mTORC1 inhibitions are protective. In particular, we provided the first evidence that genetic mTOR inhibition is protective during myocardial ischemia. Thus, the function of mTORC1 in CMs appears to be context dependent. mTORC1 activation might be required for cell growth in response to mechan-

Rheb inhibition during energy deprivation is required for autophagy activation, which is protective in this condition. In fact, suppression of autophagy by knockdown of Beclin-1 completely abrogated the protective effect of Rheb knockdown in CMs during GD. Restoration of autophagy through treatment with trehalose or overexpression of Atg7, which stimulates autophagy through mTOR-independent mechanisms, significantly reduced CM death induced by forced Rheb activation. Therefore, although it is still debated whether autophagy is protective or detrimental during cardiac stress, we have demonstrated that Rheb-regulated autophagy is protective during CM nutrient starvation and ischemia. In particular, we showed that Rheb-regulated autophagy is protective through the preservation of ATP content and reduction of misfolded protein accumulation, namely ER stress.

Rheb-induced inhibition of autophagy was accompanied by downregulation of Atg7 protein levels. Overexpression of Atg7 was sufficient to restore autophagy and to suppress Rheb-induced cell death during GD, suggesting that Rheb regulates autophagy, in part, through Atg7. mTORC1 was suggested to modulate autophagy through Ulk1/2 regulation. The role of Ulk1/2 in mediating expression of Atg7 remains to be elucidated.

Interestingly, inadvertent activation of the Rheb/mTORC1 pathway is observed in HFD-induced obesity. Obesity is characterized by glucose intolerance and dyslipidemia, and it is associated with an increased susceptibility to myocardial ischemia. We demonstrated that autophagy is reduced in the hearts of mice with HFD-induced obesity. These mice exhibited exacerbated myocardial injury in response to prolonged ischemia, which was normalized by rapamycin treatment or genetic mTOR inhibition, suggesting that increased mTORC1 activity may be responsible for the increased susceptibility. Remarkably, inhibition of Beclin-1 was associated with the failure of pharmacological mTORC1 inhibition to reduce ischemic injury in HFD mice, indicating that reactivation of autophagy is the crucial mechanism mediating the beneficial effects of mTORC1 inhibition in HFD-induced obesity.

Severe obesity and metabolic syndrome are associated with increased cardiovascular risk events and a poor prognosis in patients after acute MI. If our results hold true in humans, it may be helpful to treat patients with obesity and metabolic syndrome by use of pharmacological inhibitors of Rheb or mTORC1 to stimulate autophagy during an acute episode of myocardial ischemia. Our results are also supported by an interesting previous study that showed that obesity increases vascular senescence and vascular dysfunction in response to mTOR activation.

Other previous studies showed increased basal mTORC1 activity in the liver, adipose tissue, vasculature, skeletal muscle, and cardiac muscle in both genetic and diet-induced models of obesity and dysmetabolic conditions. AMPK inhibition has been proposed as the main intracellular mechanism leading to mTORC1 activa-
tion.

Our study extends this previous evidence, suggesting that Rheb is involved in the activation of mTORC1 induced by AMPK downregulation.

Several stimuli may enhance the activity of the Rheb/mTORC1 pathway in the tissues of obese and dysmetabolic animals. High caloric intake may represent one possible cause. High levels of circulating and cardiac lipids may also represent potential mechanisms. In addition, increases in circulating insulin, amino acids, cytokines, and adipokines may contribute to the increased Rheb/mTORC1 activity in HFD mice.

In summary, our study demonstrates that inactivation of Rheb protects CMs during energy deprivation through activation of autophagy, reduction of energy expenditure, and attenuation of ER stress (online-only Data Supplement Figure XllE). Rheb and mTORC1 may represent therapeutic targets to reduce myocardial damage during acute myocardial ischemia, particularly in patients with obesity and metabolic syndrome.

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Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

The incidence of heart failure after acute myocardial infarction (MI) remains very high in patients. This highlights the necessity to clarify the mechanism regulating the survival and death of cardiomyocytes in response to ischemia and to find new cardioprotective therapies reducing ischemic injury. We discovered that Rheb, a small GTP-binding protein, plays a pivotal role in regulating the survival of cardiomyocytes during prolonged myocardial ischemia. Rheb activity is reduced in the ischemic heart, thereby causing the suppression of the mTORC1 pathway. Inhibition of the Rheb/mTORC1 pathway is an adaptive response during ischemia, because forced restoration of cardiac Rheb activity is detrimental under this condition. Rheb inhibition is required for the activation of autophagy, an intracellular degradation process for proteins and organelles, which is protective during energy stress through preservation of cellular energy and relief of ER stress. We discovered that obesity and metabolic syndrome (Ob/MS) are associated with cardiac activation of Rheb/mTORC1 at baseline and during ischemia. In obese mice, autophagy in the heart was suppressed and ischemic injury was exacerbated. Remarkably, inhibition of mTORC1 restores autophagy and reduces infarct size in these animals after prolonged ischemia. Thus, our results suggest that Rheb and mTORC1 may be promising therapeutic targets to reduce myocardial damage after prolonged ischemia in patients with Ob/MS who display deregulated activation of the Rheb/TORC1 pathway and consequent inhibition of autophagy.
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Supplemental Methods

Primary cultures of neonatal rat ventricular cardiomyocytes and reagents

Primary cultures of ventricular cardiomyocytes were prepared from 1-day-old Crl: (WI) BR Wistar rats (Charles River Laboratories). A cardiomyocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient. Cells were cultured in a complete medium (cardiomyocyte) containing Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 5% horse serum, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite (Life Technologies, Inc.), 2 g/liter bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 μM ascorbic acid, 100 μg/ml ampicillin, 5 μg/ml linoleic acid and 100 μM 5-bromo-2'-deoxyuridine (Sigma). Trehalose and rapamycin were purchased from Sigma.

Generation of Tg-Rheb mice

A tetracycline-responsive binary α-MHC transgene system was used to allow temporally regulated expression of wild-type Rheb in cardiomyocytes \(^1\). Mice were generated on a FVB background. Doxycycline was administered in the food with a special diet formulated by Purina (625 mg/kg in pellets). In the experiments that required Rheb protein induction, doxycycline was removed from the food at 3-4 weeks of life, resulting in induced expression of Rheb a few weeks later. All experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

Construction of adenoviruses

Recombinant adenovirus vectors were constructed as described \(^2\). pBHGlaxΔE1,3Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC shuttle vector containing the gene of interest into 293 cells. We made replication-defective human adenovirus type 5 (devoid of E1) harboring full length wild-type Rheb cDNA (Ad-Rheb) and full length wild-type Atg7 (Ad-Atg7), and
we used adenovirus harboring beta-galactosidase (Ad-LacZ) as a control. For the construction of short hairpin RNA (sh-RNA) adenoviral expression vectors, pSilencer 1.0-U6 expression vector was purchased from Ambion. The U6 RNA polymerase III promoter and the polylinker region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). The hairpin-forming oligos of Rheb, Beclin-1, Raptor, Rictor and TSC2 rat cDNA and their antisenses with ApaI and Hind III overhangs were synthesized, annealed and subcloned distal to the U6 promoter. Recombinant adenoviruses were generated using homologous recombination in 293 cells. Cardiomyocytes were transduced with adenovirus for 48 hours in case of overexpression, and for 96 hours for sh-RNA-mediated knock-down.

**Echocardiography**

Echocardiography was performed after mice were anesthetized with 12 μl/g body weight of 2.5% Avertin, as described previously².

**Prolonged ischemia**

Pathogen-free mice were housed in a temperature-controlled environment with 12 hr light/dark cycles, where they received food and water ad libitum. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc) was used with 65% oxygen during the surgical procedure. The animals were kept warm using heat lamps and heating pads. Rectal temperature was monitored and maintained between 36.5 and 37.5°C. The chest was opened by a horizontal incision through the muscle between the ribs (third intercostal space). Ischemia was achieved by ligating the anterior descending branch of the left coronary artery (LAD) using an 8-0 nylon suture, with a silicon tubing (1 mm OD) placed on top of the LAD, 2 mm below the border between left atrium and left ventricle (LV). Regional ischemia was confirmed by ECG change (ST elevation).

**Assessment of area at risk and infarct size**
After 3 hours of ischemia, the animals were reanesthetized and intubated, and the chest was opened. After arresting the heart at the diastolic phase by KCl injection, the ascending aorta was cannulated and perfused with saline to wash out blood. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 10 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured using ImageJ (NIH) and Adobe Photoshop (Adobe Systems Inc.), and the values obtained were averaged. The percentages of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct area/AAR were expressed as percentages. The extent of necrosis after 30 minutes of ischemia was quantified through Hairpin-2 staining (See below), since TTC staining is less accurate for the assessment of infarct size after this period of time³.

**High fat diet (HFD) mice feeding**

C57BL/6J wild-type mice, heterozygous Beclin-1 systemic knock-out mice, conditional mTOR knock-out mice crossed with α-MHC-MerCreMer mice (C57BL/J background), and heterozygous GFP-LC3 transgenic mice (C57BL/6J background, strain GFP-LC3#53, RIKEN BioResource Center) containing a rat LC3-GFP fusion under control of the chicken β-actin promoter, were fed ad libitum with HFD (Research Diets D12492) for 18-20 weeks. A control group of mice matched for age and gender was fed with control diet for the same period of time.

**Mice hematochemical analysis**

Serum levels of glucose, cholesterol, triglycerides and non-esterified fatty acids (NEFA) in mice fed with control diet and high-fat diet were assessed by *in vitro* enzymatic colorimetric methods (Wako), according to the manufacturer’s instructions. Serum levels of insulin were assessed with an ELISA assay (Crystalchem).
Evaluation of apoptosis in tissue sections

DNA fragmentation was detected *in situ* using the TUNEL assay, as described previously. Briefly, deparaffinized sections were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP using TdT (Roche Molecular Biochemicals). Nuclear density was determined by manual counting of DAPI-stained nuclei in six fields for each animal using the 40x objective, and the number of TUNEL-positive nuclei was counted by examining the entire section using the same power objective.

**TUNEL staining in cultured cardiomyocytes**

Myocytes were fixed in PBS containing 4% paraformaldehyde. Staining was performed using the In Situ Cell Death Detection kit (Roche).

**Assessment of necrosis *in vivo* with Hairpin-2 staining**

A double-stranded DNA fragment with blunt ends was prepared as previously described. Polymerase chain reaction (PCR) with Pfu Ultra polymerase was performed with 16.6 μM Texas Red-12-dUTP (Molecular Probes), 16.6 μM dTTP, 50 μM dATP, 50 μM dCTP and 50 μM dGTP. The Pfu probe recognizes a form of DNA damage characterized by cleavage of multiple DNA fragments with blunt ends, typically observed in necrotic cell death. Heart sections were deparaffinized with xylene, rehydrated in graded alcohol concentrations, briefly washed in water and then treated with proteinase K (50 μg/ml) in PBS for 45 minutes at 37°C. After washing with PBS, a mix of 50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 10 mM, DTT, 1 mM ATP, 25 μg/ml BSA, 15% polyethylene glycol (8,000 mol wt, Sigma), 1 μg/ml Texas red-labeled DNA fragment and 250 U/ml DNA T4 ligase (Roche) was added. Sections were then placed in a humidified box for 16 hours. The sections were thoroughly washed in 70°C water and observed under a fluorescent microscope immediately after counterstaining with 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI).
Cell size evaluation and histological analysis

Heart specimens were fixed with 10 % neutral buffered formalin, embedded in paraffin, and sectioned at 6-μm thickness. Cell size was assessed through wheat germ agglutinin staining, as previously described. Immunofluorescent staining was performed with reagents and protocols previously described. Cardiac myocytes were stained with anti-troponin T antibody. Alexa 488- and Alexa 594-conjugated secondary antibodies (Invitrogen) were used. Nuclei were stained with DAPI.

Viability of the cells

Viability of the cells was measured by Cell Titer Blue (CTB) assays (Promega). In sum, cardiomyocytes (1x10^5 per 100 μl) were seeded onto 96-well dishes. After 24 hours, the medium was changed to a serum free medium. Cardiomyocytes were transduced with adenovirus harboring Rheb, Atg7 or LacZ for 36-48 hours, or shRNA against Rheb, TSC2, Beclin-1 or scramble shRNA for 96 hours, and then changed to a glucose free medium for the time required by the experiment. Viable cell numbers were measured by the CTB assay. The CTB assays were performed according to the supplier's protocol. In vitro, necrosis was assessed through propidium iodide staining as previously described.

Evaluation of autophagy

Autophagy was assessed by three different methods: LC3-II accumulation, p62 accumulation and autophagosome formation. In vivo, LC3-II accumulation was evaluated in the early phase of ischemia (30 minutes), since LC3-II may be degraded and may not be reliable in the later phase. Autophagy in the later phase of ischemia was assessed by accumulation of p62, a protein known to be degraded by autophagy, since p62 accumulation represents a more stable marker of autophagy. Autophagosome formation in vivo was evaluated by counting GFP-LC3 dots in at least seven independent fields of heart sections from Tg-GFP-LC3 mice. These mice selectively express LC3
conjugated to a green fluorescent protein in the heart. GFP-LC3 dots were also evaluated after chloroquine administration (10 mg/kg i.p.) to evaluate autophagic flux, as previously described.

For analysis of autophagosome formation *in vitro*, cardiomyocytes were grown on gelatinized coverslips. Myocytes were transduced with Ad-GFP-LC3, viruses that express GFP-LC3, for 48 hours. Samples were mounted using a SlowFade Light Antifade Kit (MolecularProbes), and the fluorescence of GFP-LC3 was observed under a fluorescence microscope. The number of cells with GFP-LC3 dots was counted in at least seven independent visual fields.

**Gene expression analysis**

mRNA expression of p62, atrial natriuretic factor (ANF) and GAPH (loading control) was evaluated with quantitative real time PCR, as described. The following primers were used: p62 sense 5'-CAGGCCGACTACCGCGATGA-3', antisense 5'-TCGCACACGCTGCACAGGTC-3'; ANF sense 5'-ATGGGCTCCTTCTCCATC-3', antisense 5'-ATCTTCTCGTACCGGAAGCTG-3'; and GAPDH sense 5'-TTCTTGTGCAGTGGCCAGCTCG-3', antisense 5'-TAGGAACACCGGAAGCCATGCGC-3';

**Immunoblot analysis, antibodies and reagents**

For immunoblot analyses, heart homogenates and cardiomyocyte lysates were prepared in a RIPA lysis buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 1 mM EDTA, 0.1 mM Na3VO4, 1 mM NaF, 50 μM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin and 5 μg/ml leupeptin. The antibodies used include anti-Rheb (Cell Signaling Technology and Santa Cruz), phospho-p70S6K (Thr389, Cell Signaling Technology), p70S6K (Santa Cruz), phospho-4E-BP1 (Thr37/46, Cell Signaling Technology), 4E-BP1 and mTOR (Cell Signaling Technology), TSC2 (Santa Cruz), p62 (American Research Products, Inc.), LC3 (MBL), Bip (Cell Signaling Technology), phospho-PERK (Thr980, Cell Signaling Technology), CHOP (Santa Cruz), Caspase-12 and cleaved Caspase-3 (Cell
Signaling Technology), Beclin-1 (Pharmingen), Atg4 (MBL), Atg5 (MBL), Atg7 (MBL), Ulk1 (Abcam), phospho-IR (Tyr11262/3, Santacruz), phospho-IRS1 (Tyr989, Ser636, Santacruz), IR and IRS-1 (Cell Signaling Technology), phospho-Akt1 (Ser473, Millipore), Akt1 (Cell Signaling Technology), GAPDH (Sigma) and tubulin (Sigma).

**Measurement of Rheb activity**

Endogenous Rheb activity is commonly evaluated by the ratio of GTP and GDP bound to Rheb, as Rheb is highly active when it is rich in GTP\(^1\). Rheb-bound GTP and GDP amounts were assessed according to a previously described luminometric method\(^12,13\). This method represents a well-established assay for the measurement of the activity of GTP-binding proteins\(^14,15\).

Proteins were extracted from cultured cardiomyocytes or mouse heart specimens in an ice-cold HEPES-based buffer containing 10 mM MgCl\(_2\), protease inhibitors and 1% Igepal CA-630\(^15\). The anti-Rheb C antibody or control IgG was added to the samples, which were shaken at 4°C overnight in the presence of 500 mM NaCl, 0.5% deoxycholate and 0.05% SDS. Protein G-agarose was then added to each sample. After shaking for 2 hours at 4°C, the agarose beads were washed four times in lysis buffer containing NaCl and detergents, and two times in 20 mM TrisPO\(_4\), 5 mM Mg\(_2\)SO\(_4\). The beads were resuspended in 20 mM TrisPO\(_4\), 1mM DTT, 1 mM EDTA, and heated at 100°C for 3 min to elute GTP and GDP bound to the immunoprecipitated Rheb. In each sample, GTP and the sum of GTP plus GDP were measured in coupled enzymatic assays\(^15\). GTP was converted to ATP by nucleoside diphosphate kinase (3 μu) in the presence of ADP (10 pmol), and the resulting ATP was measured by the luciferase method (4 mmol of luciferin and 8x10\(^8\) light units of luciferase). The sum of GTP plus GDP was measured by converting GDP to GTP using pyruvate kinase (3 μu) and phosphoenolpyruvate (50 μM). GTP, which at this point represents the sum of GDP plus GTP, was measured as described above. The GTP/GTP+GDP ratio was then calculated by the ratio of the emitted light in the two reactions.

**Measurement of intracellular ATP content**
Intracellular ATP content was measured using an ATP Bioluminescent Assay Kit (Sigma). Cells were scraped with PBS, and then half of them were used for protein content assay and the other half for ATP content measurement. For the latter assay, cells were lysed in the somatic-cell ATP-releasing agent, and the lysates were assayed according to the manufacturer's instructions, using a 1:625 dilution of the ATP assay mix. Light emitted was measured using a luminometer and was then normalized for protein content of the sample. For ATP content assays in myocardial tissue, heart specimens of equal weights were directly lysed in the somatic-cell ATP-releasing agent, and the lysates were assayed as described above.
Supplemental Tables

Table I. Echocardiographic parameters of Tg-Rheb.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NTg (N=4)</th>
<th>Tg-Rheb (N=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWTd (mm)</td>
<td>0.91 ± 0.05</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.89 ± 0.10</td>
<td>3.92 ± 0.11</td>
</tr>
<tr>
<td>PWTd (mm)</td>
<td>0.87 ± 0.05</td>
<td>0.89 ± 0.03</td>
</tr>
<tr>
<td>SWTs (mm)</td>
<td>1.48 ± 0.11</td>
<td>1.51 ± 0.09</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.31 ± 0.11</td>
<td>2.29 ± 0.05</td>
</tr>
<tr>
<td>PWTs (mm)</td>
<td>1.21 ± 0.05</td>
<td>1.30 ± 0.08</td>
</tr>
<tr>
<td>FS (%)</td>
<td>40.49 ± 1.23</td>
<td>41.38 ± 1.05</td>
</tr>
</tbody>
</table>

SWTd: diastolic septum wall thickness; LVEDD: left ventricular end-diastolic diameter; PWTd: diastolic posterior wall thickness. Data is presented as (mean ± SEM).
Table II. Body weight and hematochemical tests in HFD mice as compared with CD mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control diet (N=15)</th>
<th>High fat diet (N=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>26.8 ± 1.0</td>
<td>54.6 ± 3.3*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>112.9 ± 6.2</td>
<td>176.0 ± 17.3*</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.23 ± 0.01</td>
<td>0.43 ± 0.02*</td>
</tr>
<tr>
<td>HOMA Index</td>
<td>1.8 ± 0.22</td>
<td>5.8 ± 0.65*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>84.1 ± 3.1</td>
<td>126.1 ± 8.0*</td>
</tr>
<tr>
<td>Tryglicerides (mg/dl)</td>
<td>36.3 ± 3.7</td>
<td>71.2 ± 9.5*</td>
</tr>
<tr>
<td>NEFA (meq/l)</td>
<td>0.93 ± 0.12</td>
<td>1.87 ± 0.35*</td>
</tr>
</tbody>
</table>

HOMA: Homeostatic Model Assessment; NEFA: not-esterified fatty acids. Data is presented as (mean ± SEM); *p<0.05.
Table III. Echocardiographic parameters of HFD mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CD (N=5)</th>
<th>HFD (N=6)</th>
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</thead>
<tbody>
<tr>
<td>Gravimetric parameters</td>
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<tr>
<td>HW/TL (mg/mm)</td>
<td>6.4 ± 0.3</td>
<td>8.2 ± 0.4*</td>
</tr>
<tr>
<td>LVW/TL (mg/mm)</td>
<td>4.1 ± 0.1</td>
<td>5.9 ± 0.4*</td>
</tr>
<tr>
<td>RVW/TL (mg/mm)</td>
<td>0.9 ± 0.03</td>
<td>1.3 ± 0.07*</td>
</tr>
<tr>
<td>Lung/TL (mg/mm)</td>
<td>7.9 ± 0.15</td>
<td>7.4 ± 0.28</td>
</tr>
<tr>
<td>Echocardiographic parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWTd (mm)</td>
<td>0.88 ± 0.03</td>
<td>1.08 ± 0.05*</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.85 ± 0.08</td>
<td>4.12 ± 0.08</td>
</tr>
<tr>
<td>PWTd (mm)</td>
<td>0.85 ± 0.05</td>
<td>1.00 ± 0.03*</td>
</tr>
<tr>
<td>SWTs (mm)</td>
<td>1.31 ± 0.04</td>
<td>1.51 ± 0.05*</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.30 ± 0.06</td>
<td>2.55 ± 0.05</td>
</tr>
<tr>
<td>PWTs (mm)</td>
<td>1.26 ± 0.04</td>
<td>1.47 ± 0.06*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>39.26 ± 1.04</td>
<td>38.61 ± 0.99</td>
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</tbody>
</table>

Control diet: CD; HFD: high fat diet; HW: heart weight; LVW: left ventricular weight; RVW: right ventricular weight; TL: tibia length; SWTd: diastolic septum wall thickness; LVEDD: left ventricular end-diastolic diameter; PWTd: diastolic posterior wall thickness. Data is presented as (mean ± SEM); *p<0.05.
**A**

![Bar chart showing PI Positive Nuclei (% of control) for LacZ, Rheb, and Sh-Rheb at GD 18 h.]

**B**

![Bar chart showing TUNEL Positive Nuclei (% of control) for Sh-CT, Sh-TSC2 at GD 10 h.]

**Fig. II**
Cells with Numerous Dots (%)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells with Numerous Dots (%)</th>
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<tbody>
<tr>
<td>Sh-CT + GD 4 h</td>
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</tr>
<tr>
<td>Sh-TSC2 + GD 4 h</td>
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</tbody>
</table>

**Fig. III**

Normalized LC3II Accumulation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normalized LC3II Accumulation</th>
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<tbody>
<tr>
<td>Sh-CT Sh-Rheb Sh-TSC2</td>
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</tr>
<tr>
<td>Sh-CT Sh-Rheb Sh-TSC2</td>
<td>P&lt;0.05</td>
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</tbody>
</table>

**D**

Cells with Numerous Dots (%)

<table>
<thead>
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<th>Condition</th>
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</thead>
<tbody>
<tr>
<td>Sh-CT</td>
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</tr>
<tr>
<td>Sh-TSC2</td>
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**F**

Cells with Numerous Dots (%)

<table>
<thead>
<tr>
<th>Condition</th>
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<tbody>
<tr>
<td>Sh-CT</td>
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<tr>
<td>Sh-Rheb</td>
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**G**

Cells with Numerous Dots (%)

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<tr>
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<tr>
<td>Sh-Rheb</td>
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</table>
A

<table>
<thead>
<tr>
<th>LacZ</th>
<th>Atg7</th>
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<tr>
<td>Atg7</td>
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<td>GAPDH</td>
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B

<table>
<thead>
<tr>
<th>LacZ</th>
<th>Rheb</th>
<th>Rheb+Atg7</th>
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<tbody>
<tr>
<td>LC3</td>
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<td>p62</td>
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<td></td>
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<tr>
<td>Tubulin</td>
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</table>

C

GD 4h

Rheb | Rheb+Atg7

D

P<0.05  P<0.05

Cells with Numerous Dots (%)

LacZ | Rheb | Rheb+Atg7

E

<table>
<thead>
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<th>Trehalose 100 mM</th>
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<td>LacZ</td>
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<tr>
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<tr>
<td>LC3</td>
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<td>p62</td>
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<td>P-p70 S6K</td>
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<tr>
<td>GAPDH</td>
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F

<table>
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<tr>
<th>Trehalose 100 mM</th>
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<tbody>
<tr>
<td>LacZ</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>Atg7</td>
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</tr>
</tbody>
</table>
A. 

Hairpin-2 Positive Nuclei (%)

CT | Tg-Rheb
---|---

30 min Ischemia

P<0.01

B. 

Control | Tg-Rheb
---|---

DAPI | Hairpin-2 | DAPI | Hairpin-2

C. 

6h Ischemia

Control | Tg-Rheb
---|---

D. 

AAR (%)

CT (n=6) | Tg-Rheb (n=7)

P<0.01

E. 

MI Size/AAR (%)

CT (n=6) | Tg-Rheb (n=7)
Fig. VIII

A

<table>
<thead>
<tr>
<th></th>
<th>CT</th>
<th>Tg-Rheb</th>
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<td>GAPDH</td>
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B

Normalized LC3II Accumulation

<table>
<thead>
<tr>
<th>Controls</th>
<th>Ischemia</th>
<th>P&lt;0.05</th>
<th>P&lt;0.05</th>
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<tbody>
<tr>
<td>CT</td>
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C

Normalized p62 Accumulation

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<tbody>
<tr>
<td>CT</td>
<td>Tg-Rheb</td>
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D

ATP Content (% of control)

<table>
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<th>Controls</th>
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<td>Baseline</td>
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<tr>
<td>Tg-Rheb</td>
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E

<table>
<thead>
<tr>
<th>PI (3 h)</th>
<th>CT</th>
<th>Tg-Rheb</th>
<th>CT</th>
<th>Tg-Rheb</th>
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<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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CHOP

Tubulin

F

Normalized CHOP Accumulation

<table>
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<tr>
<td>CT</td>
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<tr>
<td>CT</td>
<td>Tg-Rheb</td>
<td></td>
</tr>
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**A**

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**B**

- **Fig. IX**

<table>
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**C**

- **Control Diet**
- **High Fat Diet**

**D**

- **ANF Expression (fold vs. control)**

- **CD**
- **HFD**
A. Graph showing relative expression level of P-p70 S6K and P-AMPK with significance levels.

B. Bar graph showing normalized LC3II accumulation with significance levels.

C. Bar graph showing normalized P62 accumulation with significance levels.

D. Images showing control diet and high fat diet conditions.

E. Graph showing dots/field (n) with significance level.

F. Western blot analysis for LC3 and GAPDH under ischemia conditions.
Supplemental Figure Legends

**Figure I.** A, Rheb expression and phosphorylation status of p70<sup>S6K</sup> were evaluated in cardiomyocytes transduced with adenovirus expressing a short hairpin sequence, targeting Rheb for 96 hours. B, Phosphorylation status of p70<sup>S6K</sup> was evaluated in cardiomyocytes transduced with increasing doses of Ad-Rheb, after 4 hours of GD. C, Rheb was overexpressed in cardiomyocytes both at baseline and during GD, and it was then immunoprecipitated. Physical interaction between Rheb and mTOR was evaluated both at baseline and during glucose deprivation. D-F, Phosphorylation status of p70<sup>S6K</sup> was evaluated in cardiomyocytes with sh-TSC2 depletion, sh-Rheb, or with sh-TSC2 (D) and sh-Rheb together (E). Densitometric quantification is also shown. N=4 (F). G-H, mTORC2 activity, as assessed by the phosphorylation status of Akt (Ser 473), was evaluated in cardiomyocytes transduced with Ad-LacZ or Ad-Rheb, both at baseline and after 4 hours of GD. N=3.

**Figure II.** A, Cardiomyocytes were transduced with Ad-LacZ or Ad Rheb for 48 hours, or with sh-Rheb for 96 hours, and then subjected to GD for 18 hours. Percentage of propidium iodide-positive nuclei with respect to control cardiomyocytes was evaluated. N=3. B, Cardiomyocytes were transduced with sh-scramble or sh-TSC2 for 96 hours and then subjected to GD for 10 hours. TUNEL staining was performed. Data is presented as a percentage with respect to cells transduced with control adenovirus. Control bar is set at 100%. * p<0.05 vs. LacZ or Sh-CT. Bar=50 µm. N=3.

**Figure III.** A-C, Cardiomyocytes were transduced with sh-scramble, sh-TSC2 or sh-Rheb for 96 hours. LC3 isoforms and p62 accumulation were then evaluated at baseline or after 4 hours of GD. Representative immunoblots are shown (A), together with densitometric analyses of LC3-II (B) and p62 (C). N=4. D-E, Cardiomyocytes were coinfected with sh-scramble or sh-TSC2 for 96 hours and adenovirus expressing GFP-LC3 for the last 48 hours. After 4 hours of GD, GFP-LC3 puncta were counted. The percentage of cardiomyocytes with GFP-LC3 dots among those transduced with sh-scramble or sh-TSC2 is shown (D, * p<0.05), together with representative images. N=5 (E, bar=10
µm). **F-G, Cardiomyocytes were coinfected with sh-scramble or sh-Rheb for 96 hours and adenovirus expressing GFP-LC3 for the last 48 hours. The baseline percentage of cardiomyocytes with GFP-LC3 dots among those transduced with sh-scramble or sh-Rheb is shown (F, * p<0.05; N=4), together with representative images (G, bar=10 µm).**

**Figure IV.** A, Atg7 expression levels were evaluated in cardiomyocytes transduced with Ad-LacZ or Ad-Atg7. B, LC3-II and p62 expression levels were evaluated in cardiomyocytes transduced with Ad-LacZ, Ad-Rheb, or with Ad-Rheb together with Ad-Atg7. C-D, Cardiomyocytes were infected with Ad-GFP-LC3 together with Ad-LacZ or Ad-Rheb or Ad-Rheb and Ad-Atg7 together. Percentage of cardiomyocytes with GFP-LC3 puncta was evaluated during GD; bar=10 µm. N=3. E-F, Expression levels of LC3-II, p62, phospho-p70S6K, Atg7 and Beclin-1 were evaluated in Ad-LacZ- and Ad-Rheb-transduced cardiomyocytes treated, or not treated, with trehalose.

**Figure V.** A-B, Normalized cardiac Rheb expression was evaluated in double transgenic mice (Rheb\(^+\) and tTA\(^+\); DTG) not treated with doxycycline, in DTG mice treated with doxycycline, and in Rheb\(^+\)/tTA\(^-\) mice. Representative immunoblot is presented (A) with expression levels quantified (B). N=3; * p<0.05 vs. Dox(+) and tTA (-). C-D, LV myocardial tissue sections from control mice and Tg-Rheb were stained with Rheb antibody (green fluorescence) and troponin T (red fluorescence), and then counterstained with DAPI (blue fluorescence) for nuclei visualization. Representative separate and merged panels from control (C) and Tg-Rheb (D) are presented. Bar= 50 µm.

**Figure VI.** A-B, LV myocardial sections of Tg-Rheb and controls were subjected to TUNEL and DAPI staining. The percentage of cells that were TUNEL positive is shown. * p<0.05; N=3. Representative images of the staining in the border zone are shown (B, bar=50 µm). C-D, Tg-Rheb presented a higher necrosis rate after ischemia. LV myocardial sections were subjected to hairpin-2 and DAPI staining. The percentage of cells that were hairpin-2 positive is shown. * p<0.05; N=3 (C).
Representative images of the staining in the ischemic and non-ischemic areas are shown (D, bar=50 µm).

**Figure VII. A-B,** Tg-Rheb and control mice were subjected to 30 minutes of ischemia. The extent of myocardial necrosis was evaluated with Hairpin 2 staining. The quantitative analysis of Hairpin 2 staining is shown in A. Representative Hairpin 2 staining is shown in B. N=4. **C-E,** Tg-Rheb and control mice were subjected to 6 hours of ischemia. Representative images of the TTC staining/alcian blue staining is shown in C. Bar=50 µm. The quantitative analysis of AAR and MI size/AAR is shown in D and E, respectively.

**Figure VIII. A-C,** Autophagy was evaluated in Tg-Rheb at baseline and after 30 minutes or 3 hours of ischemia, as assessed by LC3-II and p62 accumulation, respectively. A representative immunoblot is presented (A), together with the densitometric analyses of LC3 (B) and p62 (C). N=4 for each group. **D,** ATP content was assessed in the ischemic area of the left ventricles after 3 hours of ischemia. Data is presented as the percentages of respective baselines. Baseline is represented as one bar arbitrarily set at 100%. * p<0.05 with respect to relevant baseline. N=5. **E-F,** Cardiac CHOP expression was evaluated in Tg-Rheb and controls at baseline and after 3 hours of ischemia. A representative blot is shown (E), together with the densitometric analysis; N=5 for each group (F).

**Figure IX. A,** Phosphorylation status of insulin receptor β (Tyr1162/3), phosphorylation of insulin receptor substrate-1 (Tyr989, Ser 636), and phosphorylation of Akt1 (Ser473) were evaluated in HFD mice and compared to control animals. **B-C,** Cell size was evaluated through WGA staining in the heart of HFD mice with respect to controls. Cell size quantification is presented as fold vs. control, which is set at 1. * p<0.05. In C, Representative images are also presented. N=4. Bar=50 µm. **D,** ANF expression resulted to be increased in HFD mice with respect to controls. Data is presented as fold vs. CD mice. * p<0.05. N=6.
**Figure X. A-B**, HFD mice presented a higher apoptosis rate in the ischemic area after ischemia. LV myocardial sections were subjected to TUNEL and DAPI staining. The percentage of cells that were TUNEL-positive is shown. * p<0.05; N=3 (A). In B, representative images of the staining in the border zone are shown. Bar=50 µm. C-D, HFD mice presented a higher necrosis rate after ischemia. LV myocardial sections were subjected to hairpin-2 and DAPI staining. The percentage of cells that were hairpin-2 positive is presented. * p<0.05; N=3. In D, Representative images of the staining in the ischemic and non-ischemic areas are shown. Bar=50 µm.

**Figure XI. A**, HFD mice present an increase in phosphorylation status of p70^{S6K} and AMPK in the heart. Representative immunoblots are presented in Fig. 7D. N=4 for each group. B-C, Myocardial autophagy in obese mice was significantly inhibited compared to that in lean mice, both at baseline and after 30 minutes or 3 hours of ischemia, as indicated by LC3-II and p62 accumulation, respectively. N=4 for each group. Representative immunoblots are presented in Figure 7F. D-E, Chloroquine (10 mg/kg) was administered to Tg-GFP-LC3 mice fed with control diet or HFD, 4 hours before surgery. After 30 minutes of ischemia, GFP-LC3 puncta were counted in LV sections. In D, representative images are presented. Bar=50 µm. In E, quantification of the number of dots/field is shown. N=3. F, Rapamycin (1 mg/kg) was administered intraperitoneally to HFD and CD mice 60 minutes before coronary ligation. After 30 minutes of ischemia, autophagy was evaluated by LC3-II accumulation in the hearts of HFD mice treated with rapamycin, with respect to HFD mice treated with vehicle.

**Figure XII. A**, Tamoxifen (30 µg/g) was administered to α-MHC-*MerCreMer-mTOR* flox/+ mice (*mTOR*+/−) and α-MHC-*MerCreMer-mTOR* +/+ mice (controls) for 7 days. Cardiac mTOR protein levels were evaluated in *mTOR*+/− and controls 3 weeks after tamoxifen administration. B-D, Cardiac phosphorylation status of p70^{S6K}, and LC3-II accumulation were evaluated in *mTOR*+/− mice and controls, fed with control diet or HFD (N=4). * p<0.05 vs. CT. E, A schematic representation of our hypothesis. Nutrient starvation and prolonged ischemia inhibit the mTORC1 pathway through Rheb.
inhibition. Rheb/mTORC1 inhibition upregulates autophagy, which increases cardioprotection. On the other hand, obesity and metabolic syndrome activate the Rheb/mTORC1 pathway during prolonged ischemia, causing a defective activation of autophagy and a reduction in cardioprotection.
Supplemental References


특히 비만을 동반한 심근허혈에서 Rheb 조절은 치료 타겟이 될 수도 있다

백상홍 교수 가톨릭대학교 서울성모병원 순환기내과

Summary

배경
심부전증의 주요 원인질환인 심근경색에서 허혈 상태 후 심근세포의 사멸 및 생존의 관련 조절기전의 정립은 새로운 치료법 개발에 기초가 된다. Ras homology enriched in brain(Rheb)은 small GTP 결합 단백질로, 세포 스트레스 자극 동안 발현하여 세포의 보호작용을 나타내지만, 암세포에서는 강력히 발현하여 스트레스의 저항 및 성장을 촉진한다. 그러나 이들 활성이 세포 스트레스 동안 보호 또는 해로운 작용을 하는지에 대하여 명확하지 않으며, 또한 심장에서 급작스러운 에너지 박탈(energy deprivation, ED)에 대한 세포의 사멸 및 생존에 관한 조절에서 어떠한 작용을 나타내는지에 대해서는 아직 불분명하다. Rheb의 활성은 Akt, AMPK(AMP-activated protein kinase), GSK3β(glycogen synthase kinase-3β)의 신호전달에 의해 조절되며, tuberous sclerosis complex protein 1/2(TSC1/2) 복합체에 의해 억제된다. Rheb은 선택적으로 mammalian target of rapamycin(mTORC1)을 활성화시키고, 이는 단백질 전사와 같은 세포신호를 조절한다. mTORC1은 세포의 에너지 스트레스 반응에서 감소되어 있으며, 이들의 활성은 단백질의 합성과 autophagy(자가포식작용) 생성을 억제하지만, mTORC1의 활성은 세포의 생존과 사멸에 관여한다. mTORC1에 의한 심근세포의 생존 및 사멸에 관한 연구는 만성 심장 재형성에서 진행되었으나, 유의한 결과를 얻지 못하였다. 심장에서의 급성 ED에서 mTORC1과 mTORC2의 조절을 통한 효과에 관한 연구는 더 진행되어야 하고, mTORC1의 심근세포의 ED 동안 조절 기전에 Rheb의 관여 여부가 불확실하다. 따라서 본 연구에서는 심근세포 기아와 허혈 동안 세포 사멸과 관련하여 Rheb 유전자 역할과 근본적인 기전에 대해, 특히, 심근세포에서 에너지 스트레스 반응 동안 Rheb에 의한 mTORC1의 조절 여부에 관한 연구를 진행하였다.
방법 및 결과

1. 심근세포의 기아상태에서 mTORC1 억제에 Rheb이 관여함

심근세포에서 Rheb이 ED의 센서로 작용하는지 여부를 검토하였는데, 포도당 박탈(glucose deprivation, GD) 동안 Rheb의 발현이 감소하였으며, p70S6K와 4EBP1의 발현이 감소하여 mTORC1이 억제됨을 확인하였다. Rheb의 억제는 p70S6K의 발현을 억제하여 Rheb이 mTORC1을 억제함을 확인하였다. GD 동안 심근세포의 생존을 증진시키는 데 중요한 기전이다.

2. GD 동안 Rheb 활성화는 심근세포가 세포사멸에 민감하게 유도하지만, 억제 시에는 보호역할

GD 동안 심근세포 생존을 조절하는 Rheb의 역할을 조사한 결과, 비록 다른 세포에서는 Rheb의 과발현이 세포의 보호작용을 나타낸다고 보고했으나, 심근세포에서는 세포사멸 및 괴사가 더 증진되었다. Rheb은 GD 동안 mTORC1을 통해 심근세포의 생존에 음성조절을 담당하고 있으며, 따라서 GD 동안 내재성 Rheb의 활성화는 심근세포의 생존을 증진시키는 데 필요한 기전이다.

3. Rheb은 심근세포의 autophagy 조절

이상 mTORC1의 억제를 통한 Rheb의 활성 억제로 GD 동안 심근세포의 보호작용을 증가시키는 기전으로 확인하였다. 이번에는 GD 동안 Rheb의 억제가 autophagy 활성화에 미치는 영향을 분석하였다. Rheb의 활성화를 통하여 autophagy의 유전자 beclin-1, ulk-1, atg4, atg7의 발현이 감소하는 것을 확인하여, 내재성 Rheb이 autophagy의 음성 조절작용(Rheb이 감소하면 autophagy가 증가함)을 담당함을 확인하였고, 또한, Rheb은 GD 동안 autophagy를 통하여 심근세포의 생존 및 사멸을 조절하였다.

4. Rheb 과발현은 GD 상태에서는 에너지 스트레스와 endoplasmic reticulum 스트레스를 증가시킨

Autophagy의 중요한 결과는 ATP contents의 복원과 protein quality control을 포함하는데, GD 동안 Rheb의 과발현은 ATP 소모를 증가시키는 반면 Rheb의 분열은 ATP contents를 증가시켰다.

5. 지속적인 심근허혈에서 Rheb 억제는 보호작용

In vivo 상에서 ED 조건에 의해 심근세포의 생존에 대한 Rheb의 역할을 조사하였는데, 허혈 동안 Rheb의 GTP 결합 형태는 현저히 감소하는 반면에 Rheb의 전체 발현은 변화가 없고, 이는 장기간 지속된 허혈이 Rheb을 불활성화시켰다. 허혈 상태에서 p70S6K의 인산화 억제를 통해 mTORC1의 활성이 억제되었다. In vivo에서 허혈 상태 동안 Rheb 억제의 유의성을 확인하기 위하여 Tg-Rheb mice를 제작 후 지속적인 허혈 동안 Rheb에 의한 mTORC1의 활성 억제가 있었고, 또한 Tg-Rheb mouse에서 3시간 동안의 허혈 자극 후 가장 큰 허혈 크기를 보였다. 심근세포에서 지속적인 허혈 자극 후, 세포사멸 및 괴사가 Tg-Rheb에서 가장 많았고, 또한 허혈 후 허혈 심근 부분의 ATP level은 대조군보다 Tg-Rheb군에서 낮았는데, 이는 지속적인 허혈 상태의 노출 동안 심근세포의 보호작용이 내재성 Rheb의 발현 억제가 중요한 역할을 담당함을 보였다.

6. 고지방 식이 비만은 Rheb 조절장애와 지속적인 허혈 상태에서 심근 인감성 증가와 연관

비만과 대사증후군은 심장질환의 발생률과 높은 상관관계를 보이며, 비만의 합병증은 다른 기관에서
mTORC1의 활성과 autophagy의 저해와 관련되었고, 비만은 Rheb 활성의 조절장애와 연관되며, 심근 허혈의 민감성 증가를 조절한다. 고지방 식이군에서 mROTC1의 활성이 처음부터 높아 일반 식이군을 한 실험군과 같은 허혈 실험에서도의 효과를 확인할 수 없었으나, 지속적인 허혈 상태 동안 Rheb과 mTORC1의 증가와 유지는 일반 식이군보다 고지방 식이군에서 높았 다. Autophagosome의 축적이 고지방 식이군에서 높았으며, Tg-Rheb mouse에 rapamycin을 투여한 결과 autophagy는 증가하였으나, 심근경색의 크기가 모두 줄어들었으며, autophagy 반응은 고지방 식이군에서 mTORC1의 조절에 긍정적인 영향을 미칠 수 있음을 시사하였다.

결론
Rheb은 에너지 스트레스의 센서로 작용하며, ED의 반응으로 심근세포 생존의 중요한 조절자로서 역할을 한다. 심근세포와 심장에서 GD와 허혈 상태 동안 mTOR의 억제를 유도하고, Rheb은 mTOR과 함께 상호작용을 하며, GD와 허혈 동안 저해하고, Rheb의 활성화는 mTORC1의 억제를 요구한다. Rheb은 CM(cardiomyocyte)에서 ED 동안 mTORC1의 중심적인 역할을 하는데, Rheb의 활성화는 GD와 지속적인 허혈 동안 세포사멸과 세포고사를 악화시키지만, Rheb의 down regulation은 GD 동안 CM의 생존을 증가시킨다. mTORC1 활성은 mechanical overload의 반응에 의한 세포성장이 요구되며, 반면에 mTORC1 억제는 ED의 반응에 대한 에너지 상태의 보존에서 중요하다. ED 동안 Rheb 억제는 autophagy의 활성화가 필요한데, 심근세포 영양부족과 허혈 동안 Rheb에 의해 조절되는 autophagy는 보호 역할을 한다. Rheb은 Atg7을, mTORC1은 Ulk1/2의 조절을 통해 autophagy를 조절할 수 있다. Rheb/mTORC1 경로 활성은 HFD(high-fat diet)-induced obesity에서 관찰되었고, HFD-induced obesity mouse의 심장에서 autophagy가 감소하였다. Autophagy의 재활성화는 HFD-induced obesity에서 mTORC1 inhibition의 좋은 영향을 조절하는 중요한 메커니즘이다.

심각한 비만과 대사증후군은 심혈관질환의 위험도 증가와 연관이 되어있는데, 만약 이 연구결과가 사람에서도 적용된다면 비만과 대사증후군 환자에 급성 심근경색에서 Rheb 또는 mTORC1의 약리학적 저해제의 사용에 의한 autophagy의 자극으로 도움이 될 것이고, Rheb과 mTORC1은 특히 비만, 대사증후군 환자에게 급성 심근혈동안 심근세포에의 피해를 감소시키기 위한 치료 타겟으로 보인다.

Commentary
이 연구의 핵심은 허혈에 따른 심근세포의 사멸 및 생존의 조절 기전을 검증하고, 새로운 심근세포의 보호 치료 기전의 규명이다. 본 논문은 지속적인 허혈 상태 동안 심근세포의 생존조절에 Rheb이 중요한 역할을 담당하고, Rheb의 억제가 autophagy의 활성 및 세포내 단백질과 세포소기관의 제거에 중요한 역할을 담당한다는 것을 확인하였다. 또한, 비만과 대사증후군이 허혈 동안 Rheb/mTORC1에 의한 심장 활성에 연관됨을 시사하였다. 비만 쥐에서 심장의 autophagy는 감소되어 있었으며, 허혈 손상은 악화되었다. 지속적인 허혈 자극에서 mTORC1의 억제는 autophagy를 증가시키고 경색 부위를 감소시키는 효과를 관찰하였으므로, 본 연구에서는 Rheb과 mTORC1은 지속적인 허혈 및 비만과 대사증후군 치료의 표적이 될 수 있음을 시사한다.
Rheb is a Critical Regulator of Autophagy During Myocardial Ischemia
Pathophysiological Implications in Obesity and Metabolic Syndrome

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Background—Rheb is a GTP-binding protein that promotes cell survival and mediates the cellular response to energy deprivation (ED). The role of Rheb in the regulation of cell survival during ED has not been investigated in the heart.

Methods and Results—Rheb is inactivated during cardiomyocyte (CM) glucose deprivation (GD) in vitro, and during acute myocardial ischemia in vivo. Rheb inhibition causes mTORC1 inhibition, because forced activation of Rheb, through Rheb overexpression in vitro and through inducible cardiac-specific Rheb overexpression in vivo, restored mTORC1 activity. Restoration of mTORC1 activity reduced CM survival during GD and increased infarct size after ischemia, both of which were accompanied by inhibition of autophagy, whereas Rheb knockdown increased autophagy and CM survival. Rheb inhibits autophagy mostly through Atg7 depletion. Restoration of autophagy, through Atg7 reexpression and inhibition of mTORC1, increased cellular ATP content and reduced endoplasmic reticulum stress, thereby reducing CM death induced by Rheb activation. Mice with high-fat diet–induced obesity and metabolic syndrome (HFD mice) exhibited deregulated cardiac activation of Rheb and mTORC1, particularly during ischemia. HFD mice presented inhibition of cardiac autophagy and displayed increased ischemic injury. Pharmacological and genetic inhibition of mTORC1 restored autophagy and abrogated the increase in infarct size observed in HFD mice, but they failed to protect HFD mice in the presence of genetic disruption of autophagy.

Conclusions—Inactivation of Rheb protects CMs during ED through activation of autophagy. Rheb and mTORC1 may represent therapeutic targets to reduce myocardial damage during ischemia, particularly in obese patients. (Circulation. 2012;125:1134-1146.)

Key Words: apoptosis • ischemia • obesity • signal transduction

Heart failure is viewed as one of the major healthcare problems worldwide, with acute myocardial infarction (MI) as the most common predisposing cause. It is fundamental to clarify the mechanisms regulating cardiomyocyte (CM) death and survival during ischemic injury to find new therapies to reduce the amount of myocardial loss after a sudden coronary occlusion.

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Ras homology enriched in brain (Rheb) is a small GTP-binding protein that has been shown to regulate the cellular stress response, both in lower organisms and in mammalian cell lines. In particular, Rheb appears to be a critical sensor of energy stress, being inactivated under this condition. Inhibition of Rheb during cellular stress promotes the upregulation of adaptive mechanisms, such as cell cycle arrest and growth inhibition, which may save energy, favor DNA repair, and thus be protective. On the contrary, Rheb is hyperactivated in cancer cells, where it promotes stress resistance and survival, and Rheb activation was found to directly inhibit apoptotic pathways induced by amino acid deprivation and genotoxic stress. Therefore, it is not clear whether Rheb activity is protective or detrimental during cellular stress. Remarkably, the role of Rheb in response to acute energy deprivation, and in regulation of cell death and survival, has never been investigated in the heart.

Rheb activity is regulated by upstream kinases, such as Akt, AMP-activated protein kinase (AMPK), and glycogen...
synthase kinase-3β, which control Rheb through direct modulation of the heterodimer composed of the tuberous sclerosis complex proteins 1 (TSC1) and 2 (TSC2). The TSC1/TSC2 complex inhibits mTOR by exerting a strong GTPase activity toward it. Rheb directly binds and selectively activates the multiprotein complex 1 of mammalian target of rapamycin (mTORC1), which in turn mediates many cellular functions, such as protein translation. mTORC1 is also inhibited in response to energy stress, and its inactivation reduces protein synthesis and upregulates autophagy. However, mTORC1 activation also promotes cell survival and inhibits apoptosis in several stress conditions, and, therefore, whether mTORC1 inhibition is detrimental or protective during cellular stress is stimulus dependent. 

The role of mTORC1 in mediating survival and death of CMs has only been investigated in models of chronic cardiac remodeling, with discordant results. Importantly, the effect of direct and selective mTORC1 versus mTORC2 modulation during CM acute energy deprivation, such as myocardial ischemia, remains to be elucidated. It is also unclear how mTORC1 is modulated during CM energy deprivation and whether Rheb, an immediate upstream regulator of mTORC1, is critically involved in such regulation in CMs. In our study, we investigated the role of Rheb in the regulation of cell death and survival during CM starvation and ischemia, and the underlying molecular mechanisms. In particular, we studied whether a direct and selective modulation of mTORC1 induced by Rheb is involved in the effects exerted by Rheb on CM survival during energy stress. Recent reports have shown that obesity and metabolic syndrome, which are characterized by an increased risk of cardiovascular mortality and increased myocardial susceptibility to ischemic injury, are associated with a hyperactivation of tissue mTORC1. Therefore, we also evaluated whether cardiac mTORC1 is activated in obesity and metabolic syndrome, whether Rheb is involved in such phenomena, and whether a deregulated activation of Rheb and mTORC1 may be responsible for the increased susceptibility to ischemia associated with these conditions.

### Methods

#### Experimental Procedures

Experimental procedures and animal models are described in the expanded Methods section in the online-only Data Supplement. Experimental procedures, heterozygous GFP-LC3 transgenic mice, beclin-1 knockout mice, and conditional mTORC1 knockout mice have also been described elsewhere. All experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

### Statistics

Data are expressed as mean±SEM. When specified in the figure legends, presentation of bar charts was standardized by control mean×100, so that the presented bars represent the mean percentage of variation±SEM, with respect to the control mean. The difference in means between 2 groups was evaluated using the t test when sample size was appropriate and the population was normally distributed; otherwise, the Mann-Whitney U test was adopted. When differences among 3 or more groups were evaluated, the one-way analysis of variance or the Kruskal-Wallis test was used. The post hoc comparisons were performed by use of the Bonferroni post hoc test or the Mann-Whitney U test with Bonferroni correction. The shown statistical significance of differences between groups was always calculated by post hoc comparisons when multiple groups were compared. Statistical analyses were performed with the use of SPSS 15.0 (SPSS Inc, Chicago, IL) and GraphPad-Prism 5.00 (GraphPad-Software, San Diego, CA). Probability values of <0.05 were considered statistically significant.

### Results

#### Rheb Mediates mTORC1 Inhibition During Starvation in CMs

To investigate whether Rheb acts as a sensor of energy deprivation in CMs, neonatal rat ventricular CMs were subjected to glucose deprivation (GD). GTP binding of Rheb was decreased significantly in response to GD (Figure 1A), indicating that Rheb is inactivated by GD. During GD, phosphorylation of p70S6k and 4E-BP1 was progressively reduced, indicating that mTORC1 was inhibited (Figure 1B). Knockdown of Rheb, with adenovirus harboring shRNA-Rheb, inhibited phosphorylation of p70S6k at baseline, suggesting that inactivation of Rheb is sufficient to inactivate mTORC1 (online-only Data Supplement Figure 1A). Transduction of CMs with adenovirus harboring wild-type Rheb abolished the GD-induced decreases in phosphorylation of p70S6k and 4E-BP1 (Figure 1C through 1E and online-only Data Supplement Figure 1B), suggesting that Rheb inactivation is required for GD-induced suppression of mTORC1. In addition, Rheb physically interacts with mTOR both at baseline and during GD (online-only Data Supplement Figure 1C), thus indicating that Rheb directly regulates mTORC1 in CMs.

Rheb is negatively regulated by the GTPase-activating protein activity of the TSC1/TSC2 complex. Downregulation of TSC2, with adenovirus harboring shRNA-TSC2 (online-only Data Supplement Figure 1D), induced phosphorylation of p70S6k, suggesting that endogenous TSC2 negatively regulates mTORC1 in CMs (online-only Data Supplement Figure 1E and IF). Activation of mTORC1 by downregulation of TSC2 was abolished in the presence of Rheb knockdown, suggesting that TSC2 regulates mTORC1 through Rheb (online-only Data Supplement Figure 1G and HH). These results suggest that GD inhibits mTORC1, but not mTORC2, by inactivating Rheb.

#### Activation of Rheb Sensitizes CMs to Cell Death During GD, Whereas Inhibition Is Protective

We then investigated the role of Rheb in regulating CM survival during GD. Although Rheb is cell protective in other cell types, CMs in which the activity of mTORC1 was normalized with overexpressed Rheb displayed decreased survival after 10 and 18 hours of GD in comparison with control virus–treated CMs (Figure 2A). CMs overexpressed with Rheb displayed significantly more apoptosis and necrosis, as assessed by TdT-mediated dUTP nick-end labeling (TUNEL) assays and propidium iodide staining, respectively.
TSC2 knockdown also decreased survival and increased apoptosis of CMs in response to GD (Figure 2D and online-only Data Supplement Figure IIB). Conversely, downregulation of endogenous Rheb increased the survival of CMs during GD and rescued the decrease in cell survival in the presence of TSC2 knockdown during GD (Figure 2E). Furthermore, selective inhibition of mTORC1, through Raptor downregulation, with adenovirus harboring shRNA-Raptor, significantly increased CM survival during GD in Rheb-overexpressing CMs. In contrast, selective mTORC2 inhibition, through Rictor depletion, did not increase survival in Rheb-overexpressing CMs during GD (Figure 2F). Collectively, these data suggest that Rheb negatively regulates CM survival during GD through mTORC1 activation. Thus, inactivation of endogenous Rheb during GD is an adaptive mechanism that promotes survival of CMs.

**Rheb Regulates CM Autophagy**

We investigated the molecular mechanism through which inactivation of Rheb protects CMs during GD. Because Rheb inactivation causes mTORC1 inhibition during GD, and because mTOR is a negative regulator of autophagy, we hypothesized that downregulation of Rheb during GD is required for stimulation of autophagy, which may be protective in this context. As shown previously, GD increased LC3-II and decreased p62, a protein degraded by autophagy, suggesting that GD activates autophagy in CMs. However, in Rheb-overexpressing CMs, LC3-II expression was lower and expression of p62 was greater, both at baseline and during GD (Figure 3A through 3C). The number of GFP-LC3 dots, an indicator of autophagosome accumulation, during GD was significantly smaller in Rheb-overexpressing CMs than in control CMs (Figure 3D and 3E). Rheb overexpression induced significant downregulation of autophagy genes, including beclin-1, ulk-1, atg4, and atg7 (Figure 3F). Conversely, downregulation of endogenous Rheb significantly increased autophagy at baseline and during GD (online-only Data Supplement Figure III). These results suggest that endogenous Rheb negatively regulates autophagy and that inactivation of Rheb is necessary and sufficient for stimulation of autophagy in CMs during GD.

**Figure 1.** mTORC1 is downregulated during GD through Rheb inactivation. A, Rheb activity was assessed by the ratio of Rheb-bound GDP to Rheb-bound GTP levels at baseline and during GD; n = 5. B, CMs were subjected to GD for different periods of time. Phosphorylation statuses of p70S6K (Thr 389) and 4E-BP1 (Thr 37/46) were evaluated. C through E, CMs were transduced with adenovirus harboring wild-type Rheb or LacZ. After 48 hours, phosphorylation statuses of p70S6K and 4E-BP1 were evaluated at baseline and after GD. Immunoblots and densitometric analyses are presented. n = 5. GD indicates glucose deprivation; CM, cardiomyocyte.
We then asked if autophagy mediates the cell-protective effect of Rheb inactivation. The protective effect of Rheb downregulation during GD was completely abrogated when Beclin-1 was downregulated with adenovirus harboring shRNA-beclin1 (Figure 3G). These results suggest that autophagy plays an important role in mediating the protective effect of Rheb inactivation during GD. Conversely, to restore autophagy during GD in Rheb-overexpressing CMs, we expressed Atg7 with adenovirus transduction (online-only Data Supplement Figure IVA). We took this approach because Atg7 is a crucial protein for autophagosome formation, because Atg7 is markedly downregulated in Rheb-overexpressing CMs, and because overexpression of Atg7 is sufficient to reinduce autophagy when autophagy is inhibited.21,22 Atg7 overexpression significantly restored autophagy during GD in Rheb-overexpressing CMs (Figure 3H). Importantly, both Atg7 expression and trehalose pre-treatment significantly increased the survival of Rheb-overexpressing CMs during GD (Figure 3H). Collectively, these results suggest that Rheb regulates the survival and death of CMs during GD through regulation of autophagy in vitro.

Rheb Overexpression Increases Energy Stress and Endoplasmic Reticulum Stress During GD

Important consequences of autophagy include restoration of ATP contents and protein quality control. Rheb overexpression during GD significantly enhanced ATP depletion, whereas Rheb disruption significantly increased ATP content (Figure 4A). Overexpression of Rheb increased GRP78, phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase and upregulation of Ccaat-enhancer-binding protein homologous protein (CHOP), and Caspase-12 (fragment), markers of endoplasmic reticulum (ER) stress, in CMs during GD (Figure 4B and 4C). Conversely, upregulation of the ER stress markers during GD was significantly attenuated in CMs in which Rheb was knocked down (Figure 4B and 4C). Restoration of autophagy in Rheb-overexpressing CMs,
through Atg7 overexpression, significantly attenuated ATP depletion (Figure 4D) and ER stress during GD, indicating that autophagy inhibition is responsible for these derangements (Figure 4E and 4F).

**Inhibition of Rheb Is Protective During Prolonged Myocardial Ischemia**

To investigate the role of Rheb in regulating CM survival and death in response to energy deprivation in vivo, we used a mouse model of prolonged ischemia, in which the left descending coronary artery was ligated for 3 hours. During ischemia, the GTP-bound form of Rheb was significantly decreased, whereas the total expression of Rheb was not altered, suggesting that Rheb is inactivated by prolonged ischemia in vivo (Figure 5A and 5B). The activity of mTORC1, as evaluated with p70S6K phosphorylation, was also decreased during ischemia (Figure 5C and 5D).

To evaluate the significance of Rheb inhibition during ischemia in vivo, we generated transgenic mice with cardiac-specific overexpression of Rheb (Tg-Rheb) with use of a Tet-off system. In these mice, expression of the Rheb transgene in the heart was induced in the absence of doxycycline. Doxycycline was administered to the mice during the gestational period and for the first 3 to 4 weeks of life to avoid...
the effect of transgene expression during cardiac development in Tg-Rheb (Figure 5E and online-only Data Supplement Figure VA and VB). Doxycycline was terminated 6 to 8 weeks before the experiment to allow full transgene expression and eliminate possible actions of doxycycline on cell death/survival. In this protocol, expression of Rheb was 2.3-fold greater in Tg-Rheb than in control littermates (Rheb+/tTA− mice). Rheb exhibited diffuse cytoplasmic distribution in CMs of both control mice and Tg-Rheb (online-only Data Supplement Figure VC and VD). Tg-Rheb presented a normal cardiac phenotype at 3 months of age (online-only Data Supplement Table I). In Tg-Rheb mice, mTORC1 activity was significantly increased, both at baseline and during prolonged ischemia, in comparison with control mice (Figure 5C and 5D), suggesting that Rheb inactivation is required for mTORC1 inhibition during prolonged ischemia. After 3 hours of ischemia, Tg-Rheb mice exhibited a significantly greater MI size than control mice (Figure 5F through 5H). The extent of CM apoptosis and necrosis after prolonged ischemia was also greater in Tg-Rheb than in controls, as evaluated with TUNEL and Hairpin-2 staining, respectively (online-only Data Supplement Figure VI). Tg-Rheb presented increased ischemic injury even after a brief period of ischemia (30 minutes), as evaluated with Hairpin-2 staining. Tg-Rheb also exhibited significantly enhanced myocardial damage even after a longer coronary occlusion (6 hours; online-only Data Supplement Figure VII).

There was less induction of autophagy in Tg-Rheb than in control mice at baseline and during ischemia, as indicated by reduced LC3-II and increased p62 accumulation (online-only Data Supplement Figure VIIA through VIIIC). Expression of p62 did not differ at the mRNA level between controls and Tg-Rheb, indicating that increased p62 accumulation was due to reduced degradation (mRNA expression in Tg-Rheb 0.96-fold versus controls, \( P<0.05 \)). The level of myocardial ATP in the ischemic area after ischemia was significantly lower in Tg-Rheb than in controls (online-only Data Supplement Figure VIIID). The level of CHOP, an indicator of ER stress, after ischemia was also significantly greater in Tg-Rheb than in control mice (online-only Data Supplement Figure VIIIE and
These results indicate that inhibition of endogenous Rheb is protective during prolonged ischemia in vivo.

To evaluate whether the deleterious effect of Rheb overexpression during prolonged ischemia is due to the lack of mTORC1 inactivation and activation of autophagy, rapamycin, a selective inhibitor of mTORC1 and stimulator of autophagy, was administered to Tg-Rheb and control mice just before the prolonged ischemia. Rapamycin inhibited mTORC1 activity and stimulated autophagy in Tg-Rheb mice after prolonged ischemia (Figure 6A). Rapamycin significantly reduced the size of MI in response to prolonged ischemia in Tg-Rheb mice in comparison with vehicle administration (Figure 6B through 6D). Rapamycin treatment also significantly reduced CHOP accumulation and caspase-3 cleavage in Tg-Rheb hearts after prolonged ischemia (Figure 6E and 6F). These results suggest that Rheb promotes myocardial injury during prolonged ischemia by stimulating mTORC1, inhibiting autophagy and stimulating ER stress.

High-Fat Diet–Induced Obesity Is Associated With Deregulation of Rheb and Increased Myocardial Susceptibility to Prolonged Ischemia

Obesity and metabolic syndrome are associated with high cardiovascular mortality and reduced cardiac function after MI. Complications of obesity are associated with deregulated mTORC1 activation and inhibition of autophagy in other organs. We therefore investigated whether obesity is associated with deregulated Rheb activation, which in turn mediates an increased susceptibility to myocardial ischemia.

To induce obesity, C57BL/6J mice were fed with high-fat diet (HFD) mice for 18 to 20 weeks. HFD mice developed obesity and exhibited a significant increase in serum levels of glucose, cholesterol, triglycerides, and nonesterified fatty acid in comparison with mice fed with control diet (CD) mice, suggesting that HFD mice develop metabolic syndrome (online-only Data Supplement Table II). Insulin levels and the HOMA index were significantly elevated in HFD mice, consistent with the notion that these
mice develop insulin resistance. HFD mice presented increases in mTOR-dependent IRS-1 phosphorylation (serine 636), which is a marker of decreased insulin sensitivity at baseline (online-only Data Supplement Figure IXA). HFD mice showed a significant increase in left ventricular mass and left ventricular wall thickness but preserved left ventricular systolic function (online-only Data Supplement Table III). Both cell size and expression of atrial natriuretic factor, a fetal-type gene, were increased, suggesting that HFD mice develop cardiac hypertrophy (online-only Data Supplement Figure IXB through IXD). After prolonged (3 hours) ischemia, HFD mice exhibited a significantly greater MI size than CD mice (Figure 7A through 7C), which was accompanied by greater numbers of TUNEL-positive and Hairpin-2-positive cells (online-only Data Supplement Figure X), signifying that HFD increases myocardial susceptibility to ischemic injury. HFD mice also presented a greater percentage of hairpin-2-positive cells with respect to control mice after 30 minutes of ischemia (11.5 ± 1.3% versus 4.2 ± 1.0%, P < 0.05).

In HFD mice, the activity of mTORC1 was greater at baseline and remained elevated during prolonged ischemia (Figure 7D and online-only Data Supplement Figure XIA). Thus, the suppression of mTORC1 in response to prolonged ischemia observed in CD mice was attenuated in HFD mice. Although the GTP-bound form of Rheb was significantly reduced during prolonged ischemia in CD mice, it was increased at baseline and not significantly diminished during prolonged ischemia in HFD mice (Figure 7E), suggesting that the activity of Rheb and mTORC1 is elevated at baseline and remains greater in HFD mice than in CD mice during prolonged ischemia. Intriguingly, the activity of AMPK, a negative regulator of the Rheb/mTORC1 pathway, was reduced in HFD mice both at baseline and during ischemia, as indicated by a reduction in its phosphorylation status. Conversely, it was activated in CD mice during ischemia (Figure 7D and online-only Data Supplement Figure XIA).

Consistent with activation of the mTORC1 pathway, autophagy in the heart was significantly suppressed in HFD mice both at baseline and during ischemia, as indicated by decreased LC3-II and increased p62 accumulation (Figure 7F, online-only Data Supplement Figure XIB and XIC). p62 mRNA expression was unchanged (0.84-fold versus CD.
Accumulation of autophagosomes, as evaluated by use of GFP-LC3 dots, was significantly less in HFD mice than in CD mice (Figure 7G and 7H). The number of GFP-LC3 dots was significantly reduced in HFD mice during ischemia, also after administration of chloroquine, which inhibits lysosomal enzyme activity. These data indicate reduced autophagosome formation in HFD mice (online-only Data Supplement Figure XID and XIE).

To investigate if deregulated activation of the mTORC1 pathway is responsible for the reduced tolerance to prolonged ischemia of HFD mice, we administered rapamycin to these animals and evaluated its effect on ischemic injury. As we observed with Tg-Rheb, rapamycin treatment increased autophagy (online-only Data Supplement Figure XIF) and significantly reduced the MI size of both HFD mice and CD mice (Figure 8A through 8C). Rapamycin administration failed to reduce ischemic injury in heterozygous beclin-1 knockout mice (beclin-1+/−), in which autophagy cannot be activated, when fed with HFD (Figure 8D and 8E). These results indicate that autophagy reactivation mediates the beneficial effect of mTORC1 inhibition in HFD mice.
To further demonstrate that deregulated mTORC1 activation increases the ischemic susceptibility of HFD mice, we subjected mice, with inducible cardiac-specific heterozygous mTOR knockout, that were fed with HFD, to prolonged ischemia. This strategy allowed us to partially inhibit the mTOR pathway and to normalize the increased activation of mTORC1 observed in HFD mice. Tamoxifen was administered to α-myosin heavy chain promoter-MerCreMer-mTOR flox/− mice (mTOR−/−) with HFD were subjected to 3 hours of ischemia. The MI/AAR ratio was evaluated. HFD indicates high-fat diet; CD, control diet; AAR, area at risk; MI, myocardial infarct; MHC, myosin heavy chain.

**Discussion**

We have demonstrated that Rheb is inhibited in response to GD and prolonged ischemia, and inhibition of Rheb, in turn, inhibits mTORC1 in CMs. Forced activation of Rheb in such conditions stimulates ATP depletion and ER stress by suppression of autophagy, thereby inducing cell death. Thus, Rheb acts as a sensor of energy stress and as a critical regulator of CM survival in response to energy starvation. 

We have shown previously that both GD and ischemia in CMs and in the heart, respectively, induce suppression of mTOR. It should be noted that mTOR is inhibited by both Rheb-dependent and -independent mechanisms. For example, several upstream kinases, including AMPK and glycogen synthase kinase-3β, which indirectly regu-
late mTOR, inhibit Rheb through phosphorylation and consequent activation of GTPase-activating protein activity in TSC2. It should be noted, however, that mTOR is also inhibited through Rheb-independent mechanisms, such as Akt-dependent phosphorylation of mTOR and PRAS40, and AMPK-dependent Raptor phosphorylation. Our results indicate that Rheb interacts with mTOR, that Rheb is inhibited during GD and myocardial ischemia, and that its inactivation is required for mTORC1 inhibition. Conversely, neither overexpression of Rheb nor GD affected the activity of mTORC2. We therefore propose that Rheb acts as a central and direct regulator of mTORC1 during energy starvation in CMs.

Our results suggest that forced Rheb activation exacerbates cell death and apoptosis during GD and prolonged ischemia, but it does not affect cell survival at baseline. On the other hand, downregulation of Rheb increased survival of CMs during GD, intimating the involvement of endogenous Rheb in the regulation of survival/death during GD and prolonged ischemia. Previous studies have indicated that Rheb promotes cell survival and inhibits apoptotic cell death in response to stress in several cancer cells. Activation of Rheb in unstressed conditions also induces hypertrophy without cell death in CMs (not shown). Thus, the function of Rheb in cells appears to be context dependent.

Importantly, we found that downregulation of mTORC1 mediates the protective effects of Rheb inhibition during energy deprivation, because depletion of Raptor but not of Rictor, which are the adaptor proteins of complex 1 and complex 2 of mTOR, respectively, increased survival of Rheb-overexpressing CMs during GD in vitro. In addition, pharmacological and genetic inhibition of mTORC1 reduced the susceptibility to ischemic myocardial damage of Rheb-overexpressing and obese mice, and a protective effect was also observed in control animals in vivo. These results suggest that Rheb is an obvious therapeutic surrogate of mTORC1, to achieve increased CM survival during energy deprivation.

The role of mTOR in regulating the stress response is poorly understood in terminally differentiated cell types such as CMs. In particular, the role of mTOR in the regulation of CM survival has been primarily investigated through indirect means, eg, the use of pharmacological inhibitors, which may have mTOR-independent effects. In addition, the role of mTOR in cardiac stress has been mostly studied in animal models of chronic ventricular remodeling in which mTORC1 is activated, whereas we observed mTORC1 inhibition during CM energy deprivation. Interestingly, mTORC1 activation has been indicated as protective during cardiac mechanical overload. On the other hand, in our study, we demonstrated that selective and direct mTORC1 activation is detrimental during acute cardiac energy deprivation, whereas both pharmacological and genetic mTORC1 inhibitions are protective. In particular, we provided the first evidence that genetic mTOR inhibition is protective during myocardial ischemia. Thus, the function of mTORC1 in CMs appears to be context dependent. mTORC1 activation might be required for cell growth in response to mechanical overload, whereas mTORC1 inhibition is important for preservation of energy status in response to energy deprivation.

Rheb inhibition during energy deprivation is required for autophagy activation, which is protective in this condition. In fact, suppression of autophagy by knockdown of Beclin-1 completely abrogated the protective effect of Rheb knockdown in CMs during GD. Restoration of autophagy through treatment with trehalose or overexpression of Atg7, which stimulates autophagy through mTOR-independent mechanisms, significantly reduced CM death induced by forced Rheb activation. Therefore, although it is still debated whether autophagy is protective or detrimental during cardiac stress, we have demonstrated that Rheb-regulated autophagy is protective during CM nutrient starvation and ischemia. In particular, we showed that Rheb-regulated autophagy is protective through the preservation of ATP content and reduction of misfolded protein accumulation, namely ER stress.

Rheb-induced inhibition of autophagy was accompanied by downregulation of Atg7 protein levels. Overexpression of Atg7 was sufficient to restore autophagy and to suppress Rheb-induced cell death during GD, suggesting that Rheb regulates autophagy, in part, through Atg7. mTORC1 was suggested to modulate autophagy through Ulk1/2 regulation. The role of Ulk1/2 in mediating expression of Atg7 remains to be elucidated.

Interestingly, inadvertent activation of the Rheb/mTORC1 pathway is observed in HFD-induced obesity. Obesity is characterized by glucose intolerance and dyslipidemia, and it is associated with an increased susceptibility to myocardial ischemia. We demonstrated that autophagy is reduced in the hearts of mice with HFD-induced obesity. These mice exhibited exacerbated myocardial injury in response to prolonged ischemia, which was normalized by rapamycin treatment or genetic mTOR inhibition, suggesting that increased mTORC1 activity may be responsible for the increased susceptibility. Remarkably, inhibition of Beclin-1 was associated with the failure of pharmacological mTORC1 inhibition to reduce ischemic injury in HFD mice, indicating that reactivation of autophagy is the crucial mechanism mediating the beneficial effects of mTORC1 inhibition in HFD-induced obesity.

Severe obesity and metabolic syndrome are associated with increased cardiovascular risk events and a poor prognosis in patients after acute MI. If our results hold true in humans, it may be helpful to treat patients with obesity and metabolic syndrome by use of pharmacological inhibitors of Rheb or mTORC1 to stimulate autophagy during an acute episode of myocardial ischemia. Our results are also supported by an interesting previous study that showed that obesity increases vascular senescence and vascular dysfunction in response to mTOR activation.

Other previous studies showed increased basal mTORC1 activity in the liver, adipose tissue, and vasculature, and skeletal muscle and cardiac muscle in both genetic and diet-induced models of obesity and dysmetabolic conditions. AMPK inhibition has been proposed as the main intracellular mechanism leading to mTORC1 activa-
tion,18–30–34 Our study extends this previous evidence, suggesting that Rheb is involved in the activation of mTORC1 induced by AMPK downregulation.

Several stimuli may enhance the activity of the Rheb/mTORC1 pathway in the tissues of obese and dysmetabolic animals. High caloric intake may represent one possible cause. High levels of circulating and cardiac lipids may also represent potential mechanisms. In addition, increases in circulating insulin, amino acids, cytokines, and adipokines may contribute to the increased Rheb/mTORC1 activity in HFd mice.17,18,28,31,32,35

In summary, our study demonstrates that inactivation of Rheb protects CMs during energy deprivation through activation of autophagy, reduction of energy expenditure, and attenuation of ER stress (online-only Data Supplement Figure XII). Rheb and mTORC1 may represent therapeutic targets to reduce myocardial damage during acute myocardial ischemia, particularly in patients with obesity and metabolic syndrome.

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Disclosures
None.

References


### CLINICAL PERSPECTIVE

The incidence of heart failure after acute myocardial infarction (MI) remains very high in patients. This highlights the necessity to clarify the mechanism regulating the survival and death of cardiomyocytes in response to ischemia and to find new cardioprotective therapies reducing ischemic injury. We discovered that Rheb, a small GTP-binding protein, plays a pivotal role in regulating the survival of cardiomyocytes during prolonged myocardial ischemia. Rheb activity is reduced in the ischemic heart, thereby causing the suppression of the mTORC1 pathway. Inhibition of the Rheb/mTORC1 pathway is an adaptive response during ischemia, because forced restoration of cardiac Rheb activity is detrimental under this condition. Rheb inhibition is required for the activation of autophagy, an intracellular degradation process for proteins and organelles, which is protective during energy stress through preservation of cellular energy and relief of ER stress. We discovered that obesity and metabolic syndrome (Ob/MS) are associated with cardiac activation of Rheb/mTORC1 at baseline and during ischemia. In obese mice, autophagy in the heart was suppressed and ischemic injury was exacerbated. Remarkably, inhibition of mTORC1 restores autophagy and reduces infarct size in these animals after prolonged ischemia. Thus, our results suggest that Rheb and mTORC1 may be promising therapeutic targets to reduce myocardial damage after prolonged ischemia in patients with Ob/MS who display deregulated activation of the Rheb/TORC1 pathway and consequent inhibition of autophagy.