Bidirectional Regulation of Nuclear Factor-κB and Mammalian Target of Rapamycin Signaling Functionally Links Bnip3 Gene Repression and Cell Survival of Ventricular Myocytes

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Background—Tumor necrosis factor-α and other proinflammatory cytokines activate the canonical Nuclear Factor (NF)-κB pathway through the kinase IKKβ. Previously, we established that IKKβ is also critical for Akt-mediated NF-κB activation in ventricular myocytes. Akt activates the kinase mammalian target of rapamycin (mTOR), which mediates important processes such as cardiac hypertrophy. However, whether mTOR regulates cardiac myocyte cell survival is unknown.

Methods and Results—Herein, we demonstrate bidirectional regulation between NF-κB signaling and mTOR, the balance which determines ventricular myocyte survival. Overexpression of IKKβ resulted in mTOR activation and conversely overexpression of mTOR lead to NF-κB activation. Loss of function approaches demonstrated that endogenous levels of IKKβ and mTOR also signal through this pathway. NF-κB activation by mTOR was mediated by phosphorylation of the NF-κB p65 subunit increasing p65 nuclear translocation and activation of gene transcription. This circuit was also important for NF-κB activation by the canonical tumor necrosis factor-α pathway. Our previous work has shown that NF-κB signaling suppresses transcription of the death gene Bnip3 resulting in ventricular myocyte survival. Inhibition of mTOR with rapamycin decreased NF-κB activation resulting in increased Bnip3 expression and cell death. Conversely, mTOR overexpression suppressed Bnip3 levels and cell death of ventricular myocytes in response to hypoxia.

Conclusions—To our knowledge, these data provide the first evidence for a bidirectional link between NF-κB signaling and mTOR that is critical in the regulation of Bnip3 expression and cardiac myocyte death. Hence, modulation of this axis may be cardioprotective during ischemia. (Circ Heart Fail. 2013;6:335-343.)

Key Words: Bnip3 ▶ cell death ▶ hypoxia ▶ mTOR ▶ NF-κB

The loss of cardiac cells by programmed death pathways is a central underlying feature of ventricular remodeling and contractile dysfunction after myocardial infarction. Previous work by our laboratory established a critical survival role for the cellular factor Nuclear Factor (NF)-κB in ventricular myocytes. In cells, NF-κB exists as an inactive dimeric complex, comprising p65 and p50 protein subunits bound to inhibitor protein of -κB (IκBα). Activation of NF-κB requires the phosphorylation-dependent degradation of IκBα, which is mediated by the IκBα kinase complex (IKK), IKKα, IKKβ, and IKKγ (NF-kappa-B essential modulator) subunits. The loss of IκBα exposes the nuclear localization motif on p65 NF-κB subunit permitting its phosphorylation and nuclear targeting. The transcriptional activity of NF-κB is modulated by site-specific phosphorylation of the p65 subunit transcriptional activation domain at serine 276, which activates NF-κB transcription. Several kinases involved in cell survival, including phosphatidylinositol 3'-kinase/Akt kinase, signal through the IKK complex to activate NF-κB. Indeed, previous work by our laboratory established IKKβ critical for Akt-mediated NF-κB activation and suppression of hypoxia-induced death of ventricular myocytes.

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Notably, the mammalian target of rapamycin (mTOR) is serine/threonine kinase activated by phosphatidylinositol 3'-kinase/Akt signaling pathway in response to mitogenic signaling. mTOR is a highly conserved serine/threonine kinase involved in vital cellular processes, including growth, gene transcription, and protein synthesis. Among the best studied properties of mTOR kinase is its involvement in protein translation and cell growth through phosphorylation of the ribosomal S6 protein kinase and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The tubersclerosis complex, TSC1 and TSC2, inhibits mTOR and cell growth. Consequently, the functional loss of TSC1/TSC2 as commonly seen in human hamartomas is associated with

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increased mTOR activity and cell growth.

Further, mTOR kinase activity is inhibited during metabolic or hypoxic stress by liver kinase B1-AMPK in response to low cellular ATP levels.

However, despite this well established and accepted paradigm for mTOR as a central regulator of cell growth, there remains a paucity of available information regarding mTOR’s role in cell survival. Further, little is known of the cellular mediators of mTOR signaling in cardiac myocytes.

Previously, we established the inducible death factor Bnip3 sufficient to induce mitochondrial perturbations resulting in cell death of ventricular myocytes during hypoxia or ischemic stress in vivo. The fact that deregulated Bnip3 expression would otherwise be lethal to cells implies that it must be under tight control. Indeed, previous work by our laboratory established that in the absence of an ischemic or hypoxic signal, Bnip3 transcription is constitutively silenced by the assembly of NF-κB-histone deacetylase inhibitory complexes on the Bnip3 promoter. However, the functional loss of NF-κB inhibitory signaling is sufficient to activate Bnip3 gene transcription and cell death even in the absence of hypoxic signal. Given the close proximity between Akt and IKKβ signaling pathways for NF-κB activation raises the interesting possibility that survival signals elicited by NF-κB may be linked to mTOR.

In this report, we provide novel evidence that mTOR kinase is a component of the canonical signaling pathway required for IKKβ-mediated NF-κB activation in ventricular myocytes. Our data further establish an operational link between the transcriptional control of the death gene Bnip3 and cell survival that is obligatorily linked to mTOR-mediated activation of NF-κB.

Methods

Cell Culture and Transfection

Postnatal rat cardiac myocytes were isolated from 1- to 2-day-old Sprague-Dawley rats and subjected to primary culture as previously described. Cardiac myocytes or IKKβ−/− were transfected with eukaryotic expression plasmids for 24 hours after plating under serum-free Dulbecco’s Modified Eagle Medium conditions using Effectene reagent (Qiagen Inc, Canada).

Luciferase Assay

Cells were transfected with a NF-κB luciferase reporter construct designated (NF-κB luc) or Bnip3 luciferase reporter construct (Bnip3luc) as previously reported, in the presence and absence of eukaryotic expression vectors encoding wild-type and mutant mTOR kinase as reported. Cells were harvested 24 hours after transfection. Luciferase activity was normalized to β-galactosidase activity to control for differences in transfection efficiency. Gal4-fusion constructs (Gal4-p65) were gifted to the applicant by Dr Albert Baldwin (University of North Carolina, Chapel Hill, NC).

Cell Viability

Postnatal ventricular cardiomyocytes were stained with the vital dyes calcein acetoxyxymethylester (calcein-AM) and ethidium homodimer-1 (Invitrogen, Canada) 2 μmol/L of each to visualize live (green) and dead (red) cells, respectively, by epifluorescence microscopy. At least >200 cells were counted from 3 independent experiments using replicates of n=3 for each condition tested. Data are expressed as mean±SE percent dead cells from control.

Western Blot Analysis

Western blot analysis was performed for protein expression on cell lysate extracted from cardiac myocytes and mouse embryonic fibroblasts as previously reported.

Protein extracts were resolved on SDS-PAGE gels transferred to nitrocellulose membranes as we previously reported. The filters were probed with primary antibodies from vendors as indicated, respectively: Cell Signaling Inc (Akt cat #9272, p-mTOR [Ser 2448] cat #2971, mTOR cat #2972, p56k cat #2211, S6K cat #2317, p70S6K cat #905, p70 S6K cat #9202, p-4E-BP-1 cat #9455, #4E-BF1 cat #9452, NF-κB p65 cat #4764S, p-p65NF-κB [Ser 276] cat #3037S, IKKβ cat #2654, TSC1 cat #4906); α- or β-Actin (Sigma); IkBα (SC-371, Santa Cruz); p65 NF-κB (SC-372G, Santa Cruz) and p-p65NF-κB Ser276 (cat #ab30623, Abcam Inc).

Bnip3 was generated in house as we reported.

Antibodies were used at 1:1000 dilution in 2% BSA containing 0.1% TBS-T overnight at 4°C. Bound proteins were detected using secondary antimurine or rabbit antibodies (Cedarlane, Canada) conjugated to horseradish peroxidase by enhanced ECL (GE Healthcare, United Kingdom).

Statistical Analysis

Data are expressed as mean±SE. Multiple comparisons between groups were tested by 1-way ANOVA. Bonferroni post hoc tests were used to determine difference among groups. Two-tailed unpaired t test was used to compare mean difference with control. Differences were considered to be statistically significant at a level of P<0.05. Data were obtained from at least n=3 to n=4 independent myocyte isolations. Statistical analysis was performed using Graph Pad InStat Software.

Results

IKKβ-Mediated NF-κB Activation by Akt and Tumor Necrosis Factor (TNF)-α Signaling Requires mTOR

To verify that Akt-mediated activation of NF-κB requires IKKβ, we assessed NF-κB activation in postnatal ventricular myocytes in the presence of a constitutively active myristoylated Akt (Akt myr) in the absence and presence of a kinase defective mutant of IKKβ (IKKβmt). As shown in Figure 1A, in contrast to vector controls cells, a significant increase in NF-κB reporter activity was observed in cardiac myocytes expressing Akt myr. Notably, Akt-mediated NF-κB activation was impaired in the presence of the kinase inactive IKKβmt. These findings are concordant with our earlier work and verify that IKKβ is essential for Akt-mediated NF-κB activation in ventricular myocytes. Notably, expression of Akt myr was unaltered in the presence of the IKKβmt, excluding the possibility that impaired NF-κB activation by Akt myr was related to inhibition of Akt myr expression by the IKKβmt (Figure 1B). Interestingly, Akt-mediated activation of NF-κB was impaired in cells treated with the mTOR kinase inhibitor rapamycin (Figure 1C). Of note, expression of Akt in cells treated with rapamycin was unaltered excluding the possibility that the impaired NF-κB activation by Akt myr was related to inhibition of Akt myr by rapamycin (Figure 1D).

These findings raise the interesting possibility that Akt may be required for activating NF-κB. To explore this possibility, we assessed whether NF-κB activity is altered in cells defective for mTOR activity treated with TNF-α. As shown in Figure 1E, a significant increase in NF-κB reporter activity was observed in cardiac myocytes stimulated with TNF-α a finding concordant with our earlier work. However, TNF-α-mediated NF-κB activation was impaired in cells treated with rapamycin (Figure 1E). Together these findings strongly suggest that mTOR may be required by IKKβ for activating NF-κB.
IKKβ Regulates mTOR Kinase

To formally test the possibility that mTOR is a component of IKKβ signaling pathway involved in NF-κB activation, we ascertained whether mTOR kinase activity is increased in ventricular myocytes by a constitutively active form of IKKβ. As shown by Western blot analysis (Figure 2A and 2B), in contrast to vector control cells, a significant increase in phosphorylation of serine-2448 of mTOR was observed in cells expressing IKKβ. Notably, this was accompanied by a corresponding increase in the phosphorylation of mTOR target proteins, including the p-70S6K, p-S6, and p-4E-BP1 compared with control cells. Importantly, IKKβ-mediated phosphorylation of mTOR, p-S6, p-70S6K, and p-4E-BP1 was suppressed by rapamycin (Figure 2A and 2B). Notably, a kinase-defective mutant of IKKβ designated (IKKβmt), previously shown by our laboratory to be defective for activating NF-κB, was similarly defective for activating mTOR (Rimpy Dhingra, PhD and Lorrie A. Kirshenbaum, PhD, unpublished data, 2012). To substantiate these findings, we assessed next whether mTOR kinase is activated by endogenous IKKβ, for these studies we assessed p-mTOR activity in cells stimulated with TNF-α, which signals through IKKβ to activate NF-κB. As shown in Figure 2C, in contrast to vehicle-treated control cells, a marked increase in p-mTOR activity was observed in cells treated with TNF-α. These findings confirm that mTOR kinase is activated by endogenous IKKβ. To further verify that IKKβ is indeed involved in regulating mTOR kinase activity, we assessed mTOR activity in cells deficient for IKKβ. As shown by Western blot analysis (Figure 2D), in contrast to wild-type control cells, a marked reduction in p-mTOR, p-S6, p-70S6K, and p-4E-BP1 was observed in IKKβ−/− cells (Figure 2D). Importantly, repletion of IKKβ into IKKβ−/− cells restored mTOR signaling proteins, comparable with that of wild-type cells (Figure 2E). Together, these findings establish that mTOR kinase is activated by IKKβ and may be part of the signaling pathway required for NF-κB activation.

NF-κB Signaling Is Dependent on mTOR

The finding that earlier work by our laboratory established IKKβ as the principle IKK for NF-κB activation in ventricular myocytes together with our findings demonstrating that IKKβ activates mTOR kinase prompted us to assess whether mTOR kinase is required for IKKβ-mediated activation of NF-κB. For these studies, we tested whether disruption or loss of mTOR kinase would impair NF-κB activation by IKKβ.
**Transcriptional Regulation of NF-κB Activity by mTOR**

To prove that mTOR is required for downstream NF-κB activation, we next tested expression levels of the p65NF-κB complex in mouse embryonic fibroblasts defective for tuberous sclerosis complex 1 (TSC1−/−). The rational for this experiment is founded on the negative regulation of cellular mTOR activity by TSC1/TSC2, hence mTOR is constitutively active in TSC1−/− cells. As shown in Figure 4A, in contrast to wild-type cells with functional TSC1, a marked increase in basal p-mTOR activity was observed in TSC1−/− cells. This coincided with a corresponding increase in p65NF-reporter gene transcription (Figure 4B)—a finding consistent with the increased basal mTOR activity in these cells. Western blot analysis of lysate derived from wild-type and TSC1−/− served as housekeeping Western blots of cardiac cell lysate derived from infected controls (Figure 4D)—a finding concordant with our data for rapamycin. Western blot analysis of cells probed for total mTOR, p70S6K, p-4E-BP1, were inhibited in cells treated with rapamycin (Figure 2A and 2B). In addition, IKKβ-mediated NF-κB activation was similarly impaired in cells after mTOR knock-down (Figure 3D)—a finding concordant with our data for rapamycin. Western blot analysis of cell lysate (Figure 3E) verifies that mTOR was indeed knock-down in cells shown in Figure 3D. These findings strongly suggest that mTOR is a component of the IKKβ-signal transduction pathway required for NF-κB activation.

**As shown in Figure 3A and 3B, a significant increase in p65 NF-κB phosphorylation and NF-κB-dependent reporter activity was observed in cells expressing IKKβ—a finding concordant with our previously published work.** Interestingly, however, in the presence of rapamycin, IKKβ-mediated NF-κB activation was blunted compared with vehicle-treated control cells. To exclude the possibility that the observed inhibition of IKKβ-mediated NF-κB activation in cells treated with rapamycin was related to alterations in the expression levels of IKKβ, we assessed IKKβ expression in cardiac myocytes in the absence and presence of rapamycin. As shown by Western blot analysis (Figure 3C), IKKβ expression in rapamycin-treated cells was indistinguishable from cells treated with vehicle alone—verifying that the impaired activation of NF-κB activity by IKKβ was not related to inhibition of IKKβ by rapamycin. Furthermore, expression levels of IxBα were indistinguishable from vector control cells, indicating that mTOR does not influence the proteasomal degradation of IxBα. Hence, IxBα is influenced neither by mTOR activation nor by its inhibition with rapamycin (Figure 3C). On the basis of these findings, we reasoned that mTOR must directly influence NF-κB activation by a mechanism downstream of IxBα. Concordant with this notion, we observed a marked increase in the phosphorylation of p65 NF-κB subunit at Ser276, which is crucial for NF-κB nuclear targeting and gene transcription (Figure 4E). These findings support our contention that NF-κB is activated by mTOR kinase in ventricular myocytes. To test this hypothesis and further explore the possibility that p65NF-κB is directly activated by mTOR, we used Gal4-fusion proteins in which the DNA binding domain of Gal4 (Gal4DB) was fused in frame to the transactivation domain of p65NF-κB subunit (Gal4-p65TA). This would allow us to assess whether associated mTOR target proteins, including p-S6, p-70S6K, and 4E-BP1, were inhibited in cells treated with rapamycin (Figure 2A and 2B).
mTOR kinase influences p65 NF-κB transcription/translation independent of DNA binding. As shown in Figure 4F, in contrast to control cells or cells expressing the Gal4DB construct alone, a significant increase in Gal4-luciferase reporter activity was observed in cells coexpressing Gal4-p65TA activator construct. Notably, Gal4-driven luciferase reporter activity was increased even further in cells expressing the Gal4-p65STA and constitutively active mTOR. Notably, shRNA directed against mTOR suppressed Gal4-p65 luciferase reporter activity. These findings substantiate that p65 NF-κB transcription is influenced by mTOR kinase.

Loss of mTOR Activity Facilitates Bnip3 Gene Upregulation

Previously, we established that p65 NF-κB promotes cell survival of ventricular myocytes by a mechanism that involves...
the transcriptional repression of the mitochondrial death gene Bnip3. On the basis of the above findings, we reasoned that loss of mTOR-regulated NF-κB activation would increase Bnip3 gene transcription and cell death. To test possibility, we assessed basal Bnip3 gene expression in the absence and presence of rapamycin. As shown in Figure 5A and 5B, a marked increase in Bnip3 reporter gene transcription and Bnip3 protein expression was observed in cells treated with rapamycin. To exclude the possibility that the observed increase in Bnip3 expression was indirectly related to spurious effects of rapamycin on the Bnip3 promoter, we further assessed Bnip3 gene transcription and cell death (Figure 5B). Notably, expression of mTOR kinase in hypoxic cells restored cell viability that was accompanied by a concomitant decline in cell viability that was accompanied by a concomitant increase in lactate dehydrogenase release. Importantly, knockdown of Bnip3 in cells defective for mTOR kinase suppressed lactate dehydrogenase release and cell death (Figure 5D–5F). These findings establish that loss of mTOR-regulated NF-κB activity promotes cell death through a mechanism dependent on Bnip3. Further, reconstitution of NF-κB activity in cells rendered defective for mTOR kinase was sufficient to suppress Bnip3 gene activation (Figure 5G). Hence, these findings functionally couple mTOR-regulated NF-κB activation to Bnip3 expression and cell survival of ventricular myocytes.

**Inhibition of mTOR Activates Bnip3 Transcription and Cell Death**

Since earlier work by our laboratory established that Bnip3 can provoke mitochondrial perturbations and cell death of ventricular myocytes,11 we tested the impact of mTOR inhibition on cell viability under basal conditions. Notably, vital staining of cells defective for mTOR displayed a modest decline in cell viability that was accompanied by a concomitant increase in lactate dehydrogenase release. Importantly, knockdown of Bnip3 in cells defective for mTOR kinase suppressed lactate dehydrogenase release and cell death (Figure 5D–5F). These findings establish that loss of mTOR-regulated NF-κB activity promotes cell death through a mechanism dependent on Bnip3. Further, reconstitution of NF-κB activity in cells rendered defective for mTOR kinase was sufficient to suppress Bnip3 gene activation (Figure 5G). Hence, these findings functionally couple mTOR-regulated NF-κB activation to Bnip3 expression and cell survival of ventricular myocytes.

**Inhibition of mTOR and NF-κB During Hypoxia Provokes Cell Death**

To test the physiological significance of our findings, we assessed the impact of mTOR on cell viability during hypoxia. Notably, in contrast to normoxic control cells, a marked reduction in IKKβ and mTOR kinase activity was observed in cells subjected to hypoxia (Figure 6A). This was also accompanied by a marked reduction in phospho-p65 NF-κB protein, increased Bnip3 expression, and cell death (Figure 6A and 6D). Importantly, expression of mTOR kinase in hypoxic cells restored nuclear targeting of p65NF-κB suppressing Bnip3 gene transcription and cell death (Figure 6B–6E). Similarly, overexpression of the p65NF-κB subunit in hypoxic cells was sufficient to
suppress Bnip3 gene activation—a finding concordant with our data for mTOR (Figure 6F). These findings support our contention that NF-κB activation down-stream of mTOR is crucial for regulating Bnip3 expression and cell survival.

These findings strongly suggest that mTOR-regulated NF-κB signaling is crucial for regulating Bnip3 gene activity and cell survival of ventricular myocytes.

**Discussion**

The signaling networks that coordinate cell survival and cell death are poorly defined in the heart. The mTOR is a highly conserved serine/threonine kinase centrally involved in vital processes, including growth, proliferation, and protein translation; however, its role in regulating survival of cardiac myocytes under normal or disease conditions has not been formally determined. In this report, we provide new evidence that mTOR is functionally linked to the canonical IKKβ-signaling pathway for NF-κB activation and cell survival of ventricular myocytes. To date, the principle function of IKKβ has been limited to exclusive activation of NF-κB through the phosphorylation-dependent degradation of IκBα. Indeed, the earlier work by our laboratory established IKKβ to be critical for NF-κB activation and cell survival by phosphatidylinositol 3’-kinase/Akt in ventricular myocytes. The fact that NF-κB and mTOR are both activated by Akt raises the possibility for cross-talk among these signaling pathways for cell growth and survival via IKKβ. Although little is known about the signaling mechanisms that regulate mTOR activity, our findings that mTOR kinase activity was increased by IKKβ is intriguing and strongly suggest that IKKβ is involved in mTOR activation. Indeed, we showed by not 1, but by 3 independent approaches that mTOR activity is regulated in a manner dependent on IKKβ in ventricular myocytes. Because protein–protein interactions were not performed here, we do not know whether the effects of IKKβ on mTOR kinase activity are direct, indirect, or involve an intermediary factor. Similarly, it remains unknown whether mTOR directly or indirectly phosphorylates the p65NF-κB unit as other kinases may be involved in this pathway. However, based on our data showing an mTOR-dependent increase in Ser276 phosphorylation of p65NF-κB together with our Gal4-fusion experiments would argue that mTOR directly activates p65 NF-κB unit.

The relationship between NF-κB and mTOR signaling pathways is profound given that NF-κB–dependent gene transcription was increased in ventricular myocytes by mTOR or cells deficient for TSC1 signaling in which mTOR is constitutively active. The fact that NF-κB activation by TNF-α, Akt, or IKKβ was impaired in cells deficient or rendered defective for mTOR signaling strongly suggests that mTOR is essentially required for activating NF-κB. Further, another salient feature of our work highlights that IκBα phosphorylation was not influenced by mTOR kinase suggesting that the observed increased NF-κB activity by mTOR likely occurs after IKKβ-mediated degradation of IκBα. Although a causal relationship between NF-κB and mTOR kinase for cell growth had been proposed, no definitive evidence was provided in cardiac

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**Figure 6.** Hypoxia-induced Bnip3 gene activation and cell death of ventricular myocytes is suppressed by mammalian target of rapamycin (mTOR). A. Western blot analysis of ventricular myocytes subjected to hypoxia (18 hours) probed for IKKβ and P-IKKβ; p-mTOR (2448), p65NF-κB (SC-372G), and p-p65NF-κB (serine 276, Abcam ab30623) and Bnip3. B. Epifluorescence microscopy of ventricular myocytes for p65-GFP during hypoxia in the absence and presence of an expression vector encoding mTOR, see Methods for details. C. Bnip3 promoter activity in the ventricular myocytes under normoxic and hypoxic condition in the absence and presence of an expression vector encoding mTOR. Data are means±SE from 3 independent experiments performed in triplicate (n=9). D, E. Representative images of vital staining of ventricular myocytes for the same conditions shown in (C). Data are expressed as means±SE percent dead from normoxic controls counting >200 cells per each condition tested from 3 random fields. The histogram represents quantitative data for (D) from 3 independent experiments. F. Bnip3 promoter activity in the ventricular myocytes under normoxic and hypoxic condition in the absence and presence of an expression vector encoding NF-κBp65. Data are expressed as means±SE from 2 independent experiments in triplicates (n=8). Statistical significance among the groups is indicated; **P<0.01 and ***P<0.001.
myocytes.26 Hence, the findings of the present study provide compelling evidence that NF-κB activity is regulated in a manner dependent on mTOR kinase. This view is supported by the increased phosphorylation and nuclear targeting of p65-NF-κB in mTOR−/− mice.22,29 and elevated Bnip3 expression levels and signals. This view is supported by the embryonic lethality of cells avert death while responding to mitogenic/hypertrophic stimulation and cell death may explain more fundamentally, how κB signaling pathway.

κB signaling pathways is underessed by the increased Bnip3 expression in cells defective for mTOR activity. Indeed, functional loss of mTOR coincided with a loss of NF-κB activity and corresponding increase in Bnip3 gene transcription, and cell death. Importantly, the loss of cell viability in cells deficient for mTOR was rescued by repletion of NF-κB activity or by inhibition of Bnip3, highlighting a functional link between mTOR and repression of Bnip3 for cell survival. This view is in complete agreement with the apparent resistance of TSC1−/− tumor cells to cell death, which express elevated levels of NF-κB from constitutive mTOR activation.

The fact that inhibiting Bnip3 in mTOR-deficient cells suppressed cell death identities Bnip3 a key down-stream target of mTOR-regulated cell death signaling. Moreover, the fact the lactate dehydrogenase release, indicative of necrotic cell injury, was suppressed by Bnip3 inhibition in mTOR-deficient cells place Bnip3 and mTOR in the same genetic pathway. Further, the relationship between mTOR and NF-κB for cell survival is concordant with the reported ability of NF-κB and NFAT to regulate cardiac growth.27,28 Hence, our data reveal a novel connection between cardiac hypertrophy and cell survival for TOR kinase that is functionally linked to the canonical IKKβ-NF-κB signaling pathway.

The ability of mTOR signaling to repress Bnip3 expression and cell death may explain more fundamentally, how cells avert death while responding to mitogenic/hypertrophic signals. This view is supported by the embryonic lethality of mTOR−/− mice22,29 and elevated Bnip3 expression levels and death in cells defective for mTOR kinase as observed in the present study. Further transgenic mice overexpressing a constitutively active mTOR were similarly protected against ischemia–reperfusion injury—a finding concordant with our present findings.30 At present, it remains unknown whether the ability of mTOR-regulated NF-κB activation for cell survival is a universally conserved feature restricted to cardiac myocytes or operates in a cell and context-specific manner given the reported ability of NF-κB to promote cell death under certain conditions.31,32 Further, the relationship between mTOR and Bnip3 expression is complex given the reported decrease in mTOR activity in cells expressing Bnip3.33 Though unproven, based on the findings of the present study, we speculate that a feedback mechanism between mTOR and Bnip3 may exist for dually regulating cell growth and death. This view is supported by the fact that autophagy which is activated as an adaptive mechanism during cellular stress is suppressed by mTOR.

Indeed, although autophagy per se was not studied here, the ability of mTOR to regulate Bnip3 gene expression is compelling and in agreement with the reported ability of Bnip3 to promote autophagy,14 which beyond a certain threshold may be maladaptive and trigger death. This notion is substantiated by the fact that knock-down of Bnip3 was sufficient to suppress hypoxia-induced cell death of ventricular myocytes.35 Hence, the suppression of Bnip3 transcription and cell death by mTOR may reflect mTOR’s ability to suppress maladaptive autophagy induced by Bnip3. This view is consistent with the increased Bnip3 expression, lactate dehydrogenase release, and cell death in cells deficient for mTOR kinase activity.

Thus, under the conditions tested, our data highlight a novel signaling pathway that functionally links mTOR kinase and Bnip3 gene expression to cell survival of ventricular myocytes via canonical IKKβ-NF-κB signaling pathway. Interventions that selectively activate IKKβ-mTOR-NF-κB signaling may prove beneficial in curtailing cell death and decline in cardiac performance during cardiac stress imposed by ischemic or hypoxic stress.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Cell death is an underlying feature of ventricular remodeling and decline in cardiac performance after myocardial infarction. The work herein describes a novel cell survival signaling pathway that mechanistically couples the cellular protein target of rapamycin kinase and the canonical IKKc-Nuclear Factor (NF)-kB signaling pathway to mitochondrial death gene Bnip3. We specifically show that target of rapamycin kinase imparts a cell survival role in ventricular myocytes during hypoxic injury by repressing the activation of Bnip3. We further demonstrate that mammalian target of rapamycin kinase activates NF-kB for cell survival. Hence, therapeutic interventions designed to selectively activate target of rapamycin kinase during hypoxic stress may prove beneficial in circumventing cell death and decline in cardiac pump function after myocardial infarction.
Bidirectional Regulation of Nuclear Factor-κB and Mammalian Target of Rapamycin Signaling Functionally Links Bnip3 Gene Repression and Cell Survival of Ventricular Myocytes

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