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.3,5 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.4 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.5

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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.6 mTOR is a highly conserved serine/threonine kinase involved in vital cellular processes, including growth, gene transcription, and protein synthesis.7 Among the best studied properties of mTOR kinase is its involvement in translation and cell growth through phosphorylation of the ribosomal S6 protein kinase and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The tuberculosis 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...
increased mTOR activity and cell growth.\textsuperscript{8,9} Further, mTOR kinase activity is inhibited during metabolic or hypoxic stress by liver kinase B1-AMPK in response to low cellular ATP levels.\textsuperscript{10} 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.\textsuperscript{11–14} The fact that deregulated Bnip3 expression is obligatorily linked to mTOR-mediated activation of NF-\(\kappa\)B suggests that mTOR may be required by IKK\(\beta\) of cardiac myocytes. Our findings concordant with our earlier work. However, TNF-\(\alpha\)—a finding concordant with our earlier work. However, TNF-\(\alpha\) signaling pathways for NF-\(\kappa\)B activation by Akt and tumour necrosis factor (TNF)-\(\alpha\) signaling requires mTOR.

**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.\textsuperscript{15} Cardiac myocytes or IKK\(\beta\) 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).\textsuperscript{15,17} Western blot analysis was performed for protein expression on cell lysate extracted from cardiac myocytes and mouse embryonic fibroblasts as previously reported.\textsuperscript{18} 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, mTOR [Ser 2448] cat #2971, mTOR cat #2972, p56\(K\) cat #2211, S6K cat #2317, p70 S6K cat #9205, p70 S6K cat #9202, p-4E-BP-1 cat #9455, #4E-BP1 cat #9452, NF-xb p65 cat #4764S, p-p65NF-xb [Ser 276] cat #3037S, IKK\(\beta\) cat #2684, TSC1 cat #4906); \(\alpha\)- or \(\beta\)-Actin (Sigma); I\(\kappa\)B (SC-371, Santa Cruz); p65 NF-\(\kappa\)B cat #4764S, p-p65NF-\(\kappa\)B p65 cat #4764S, p-p65NF-xb Ser276 (cat #ab30623, Abcam Inc). Bnip3 was generated in house as we reported.\textsuperscript{11} 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**

**IKK\(\beta\)-Mediated NF-xb Activation by Akt and Tumor Necrosis Factor (TNF)-\(\alpha\) Signaling Requires mTOR**

To verify that Akt-mediated activation of NF-xb requires IKK\(\beta\), we assessed NF-xb 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 IKK\(\beta\) (IKK\(\beta\)mt).\textsuperscript{17} As shown in Figure 1A, in contrast to vector controls cells, a significant increase in NF-xb reporter activity was observed in cardiac myocytes expressing Akt myr. Notably, Akt-mediated NF-xb activation was impaired in the presence of the kinase inactive IKK\(\beta\)mt. These findings are concordant with our earlier work and verify that IKK\(\beta\) is essential for Akt-mediated NF-xb activation in ventricular myocytes.\textsuperscript{19} Notably, expression of Akt myr was unaltered in the presence of the IKK\(\beta\)mt, excluding the possibility that impaired NF-xb activation by Akt myr was related to inhibition of Akt myr expression by the IKK\(\beta\)mt (Figure 1B). Interestingly, Akt-mediated activation of NF-xb 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-xb activation by Akt myr was related to inhibition of Akt myr by rapamycin (Figure 1D). These findings raise the interesting possibility that mTOR may be required for activating NF-xb. To explore this possibility, we assessed whether NF-xb activity is altered in cells defective for mTOR activity treated with TNF-\(\alpha\). As shown in Figure 1E, a significant increase in NF-xb reporter activity was observed in cardiac myocytes stimulated with TNF-\(\alpha\)—a finding concordant with our earlier work. However, TNF-\(\alpha\)-mediated NF-xb activation was impaired in cells treated with rapamycin (Figure 1E). Together these findings strongly suggest that mTOR may be required by IKK\(\beta\) for activating NF-xb.
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 down-stream of TNF-α. 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, depletion 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 myocytes17 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.
α-proteins, β-ern blot analysis of wild-type (Wt) and IKK (10 nM) probed for phospho and total mTOR. Western blot analysis of lysate derived from cardiac cells stimulated with tumor necrosis factor αP70S6K, p-S6 (ser 235/236).

C, Western blot analysis of wt in the βblots of cardiac cell lysate derived from virally infected controls concordant with our previously published work.17 Interest-

βB phosphorylation and NF-κB signaling in ventricular myocytes. Figure 2.

Figure 2. IKKβ regulates mammalian target of rapamycin (mTOR) signaling in ventricular myocytes. A, Representative Western blots of cardiac cell lysate derived from virally infected controls or cells infected with an adenovirus encoding IKKβ in the absence and presence of rapamycin (100 nM). The filter was probed with antibodies directed against p-mTOR (ser 2448), p70 S6K, p-S6, p-4E-BP1, 4E-BP1.

B, Western blot analysis of cells derived from the conditions of (A) probed for total mTOR, p70 S6K,S6, p-4E-BP1, 4E-BP1. C, Western blot analysis of lysate derived from cardiac cells stimulated with tumor necrosis factor (TNF)-α (10 nM) probed for phospho and total mTOR. D, Western blot analysis of wild-type (Wt) and IKKβ−/− mouse embryonic fibroblasts (MEFs) probed for p-mTOR (ser 2448), p70S6K (Thr 389), p-S6 (ser 235/236), IKKβ, p-4E-BP1. E, Western blot analysis of IKKβ−/− MEFs reconstituted with IKKβ expression vector and probed for p-mTOR (2448) and associated target mTOR proteins, α-Actin served as a loading control.

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.17 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 IκBα were unaltered in cells treated with rapamycin (Figure 3C), excluding the possibility that rapamycin influences IKKβ-mediated NF-κB activation by altering IκBα expression. Moreover, Western blot analysis verified that mTOR and associated mTOR target proteins, including p-S6, p-70S6K, and 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-signaling pathway required for NF-κB activation.

Transcriptional Regulation of NF-κB Activity by mTOR

To prove that mTOR is required for down-stream NF-κB activation, we next tested expression levels of the p65NF-κB in mouse embryonic fibroblasts defective for tubersclerosis complex 1 (TSC1−/−).22 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 house keeping control to verify TSC1−/− are indeed deficient for TSC1 (Figure 4A). To test the possibility that NF-κB is regulated by mTOR, we assessed whether a constitutively active form of mTOR would influence NF-κB activity. As shown in Figure 4C, in contrast to vector control cells, a marked increase in NF-κB reporter activity was observed in cells expressing the constitutively active mTOR. Importantly, the increased NF-κB reporter activity was attenuated with rapamycin, or with shRNA directed against mTOR, verifying that mTOR kinase is required for NF-κB activation (Figure 4C). Because earlier work by our laboratory established that the site-specific phosphorylation of IκBα by IKKβ is a critical first step for the proteasomal degradation of IκBα and NF-κB activation,16 we assessed whether the observed increase in NF-κB activity in mTOR-activated cells was related to alterations in the expression levels of IκBα. As shown by Western blot analysis (Figure 4D) expression levels of IκBα in the absence and presence of the constitutively active mTOR were indistinguishable from vector control cells, indicating that mTOR does not influence the proteasomal degradation of IκBα. Hence, IκBα 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 activity by a mechanism downstream of IκBα. 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,13 (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...
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-p65TA 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 protein expression in cells in the presence of shRNA directed against mTOR. As shown in Figure 5C, endogenous Bnip3 protein expression in cells after mTOR knock-down was increased in cells treated with rapamycin in the absence or presence of shRNA directed against Bnip3 (Bnip3 shRNA) treated with rapamycin. The histogram represents quantitative data shown in (D). Data are presented as means±SE from at least n=3 independent experiments counting >200 cells per condition tested from 3 random fields. F, Lactate dehydrogenase release from cardiac myocytes for the conditions shown in (D). Data are mean±SE from 2 independent experiments performed in triplicate (n=6). G, Bnip3 gene transcription in cardiac cells expressing vectors encoding either control or NF-κBp65 expression vectors in the absence and presence of rapamycin (100 nM). Data are mean±SE from 3 independent experiments performed in triplicate (n=9). Statistical significance among the groups is indicated: *P<0.05, **P<0.01, and ***P<0.001.

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, 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, knock-down 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 are 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 in the absence and presence of an expression vector encoding NF-κBp65. Data are expressed as mean±SE from 2 independent experiments performed in triplicates (n=8). Statistical significance among the groups is indicated; **P<0.01 and ***P<0.001.

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
myocytes.26 Hence, the findings of the present study provide compelling evidence that NF-xB activity is regulated in a manner dependent on mTOR kinase. This view is supported by the increased phosphorylation and nuclear targeting of p65NF-xB subunit by mTOR.

Earlier work by our laboratory established a cytoprotective role for NF-xB for averting cell death of ventricular myocytes during hypoxia.28 Notably, we determined that NF-xB activation was crucial for suppressing mitochondrial perturbations and cell death of ventricular myocytes by repressing transcription of the death gene Bnip3. Hence, the unique relationship between mTOR and NF-xB signaling pathways is underscored by the increased Bnip3 expression in cells defective for mTOR activity. Indeed, functional loss of mTOR coincided with a loss of NF-xB 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-xB 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-xB from constitutive mTOR activation.

The fact that inhibiting Bnip3 in mTOR-deficient cells suppressed cell death identifies 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 cell lines Bnip3 and mTOR in the same genetic pathway. Further, the relationship between mTOR and NF-xB for cell survival is concordant with the reported ability of NF-xB 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-xB signaling pathway.

The ability of mTOR signaling to repress Bnip3 expression and cell death may explain more fundamentally, how mTOR activity. Indeed, functional loss of mTOR coincided with a loss of NF-xB 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-xB 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-xB from constitutive mTOR activation.

The fact that inhibiting Bnip3 in mTOR-deficient cells suppressed cell death identifies 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 cell lines Bnip3 and mTOR in the same genetic pathway. Further, the relationship between mTOR and NF-xB for cell survival is concordant with the reported ability of NF-xB 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-xB signaling pathway.

The ability of mTOR signaling to repress Bnip3 expression and cell death may explain more fundamentally, how mTOR regulates Bnip3 expression in cells expressing Bnip3.

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.15 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-xB signaling pathway. Interventions that selectively activate IKKβ-mTOR-NF-xB 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.

References


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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 IKKalpha-Nuclear Factor (NF)-xB 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-xb 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.
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