p90RSK Targets the ERK5-CHIP Ubiquitin E3 Ligase Activity in Diabetic Hearts and Promotes Cardiac Apoptosis and Dysfunction

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Rationale: Cardiomyocyte apoptosis is one of the key events in the development and progression of heart failure, and a crucial role for ICER (inducible cAMP early repressor) in this process has been previously reported. ERK5 is known to inhibit cardiac apoptosis after myocardial infarction (MI), especially in hyperglycemic states, via association with CHIP ubiquitin (Ub) ligase and subsequent upregulation of CHIP ligase activity, which induces ICER ubiquitination and subsequent protein degradation. The regulatory mechanism governing ERK5/CHIP interaction is unknown.

Objective: We previously demonstrated increased p90RSK activation in the diabetic heart. As a logical extension of this work, we now investigate whether p90RSK activation inhibits ERK5-mediated CHIP activation, and subsequently increases ICER levels and apoptosis.

Methods and Results: p90RSK activation inhibits ERK5/CHIP association and CHIP Ub ligase activity. p90RSK and CHIP share a common binding site in the ERK5 C-terminal domain (aa571–807). Overexpression of either p90RSK or an ERK5 fragment (aa571–807) inhibits ERK5/CHIP association, suggesting that p90RSK and CHIP competes for ERK5 binding and that p90RSK activation is critical for inhibiting ERK5/CHIP interaction. We also identified ERK5-S496 as being directly phosphorylated by p90RSK and demonstrated that an ERK5-S496A mutant significantly impairs Angiotensin II–mediated inhibition of CHIP activity and subsequent increase in ICER levels. In vivo, either cardiac-specific depletion of ERK5 or overexpression of p90RSK inhibits CHIP activity and accelerates cardiac apoptosis after MI—a phenomenon fully reversible by activating ERK5.

Conclusions: These data suggest a role for p90RSK in inhibiting CHIP activity and promoting cardiac apoptosis through binding to and phosphorylation of ERK5-S496. (Circ Res. 2012;110:536-550.)

Key Words: MAP kinase pathway ▫ ubiquitin ▫ diabetes mellitus ▫ myocardial infarction ▫ apoptosis

Diabetes mellitus (DM) is an independent risk factor for both mortality and morbidity after myocardial infarction (MI).1,2 Previously, we have reported that activation of ERK5, an atypical mitogen activated protein kinase with transcriptional activity,3–6 inhibits apoptosis and left ventricular (LV) dysfunction in DM mice after MI. Sustained elevation of inducible cAMP early repressor (ICER), a proapoptotic transcriptional repressor,7,8 favors apoptosis through inhibition of the cAMP response element binding protein (CREB)-mediated transcription and downregulation of Bcl-2.9,10 The protein level of ICER is regulated by CREB-dependent ICER gene transcription as well as proteasome-dependent ICER protein ubiquitination and degradation.11 We reported that ICER levels were significantly...
increased in diabetic mice after MI (DM+MI), and this increase in ICER levels was blunted in transgenic mice expressing a cardiac-specific constitutively active form of MEK5α (CA-MEK5α-Tg). This finding provides a mechanistic framework for understanding the cardioprotective action of ERK5 in DM+MI mice via downregulation of ICER levels and inhibition of apoptosis.

In a subsequent study we demonstrated that ubiquitination was a critical regulatory mechanism linking ERK5 activation and ICER reduction in DM mice. ERK5 positively regulated chaperone-dependent E3 ubiquitin (Ub) ligase CHIP (carboxyl terminus of Hsp70-interacting protein)-mediated ICER ubiquitination and subsequent protein degradation. This downregulation of ICER levels leads to maintaining Bcl-2 at high levels, and protects cells from apoptosis. Our studies showed that cardiac CHIP Ub ligase activity was significantly decreased after DM+MI and this decrease was rectified in CA-MEK5α-Tg mice. However, the regulatory mechanism underlying this reduction of CHIP Ub ligase activity in DM+MI mice and how ERK5 activation reversed this phenomenon remains unclear.

In this study, we show that the ERK5-CHIP module is one of the major targets of p90RSK in diabetic hearts. Our data strongly suggest that the activation of p90RSK abrogates ERK5-mediated CHIP Ub ligase activation, and accelerates apoptosis and cardiac dysfunction in the DM+MI condition.

**Methods**

Additional information regarding animals, antibodies and reagents, plasmids, adenovirus construction, cell culture, mammalian 2-hybrid analysis, immunoprecipitation, western blotting, p90RSK in vitro kinase assay, in vitro ubiquitination assay with GST-ICER, permanent coronary ligation surgery, streptozotocin (STZ) injection, apoptosis assay, echocardiographic analysis, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis of ERK5 phosphorylation by p90RSK, and statistics can be found in the online-only Data Supplement.

**Results**

**Angiotensin II Upregulates the ICER Protein Levels and Promotes Apoptosis of Cardiomyocytes via P90RSK Activation**

Angiotensin II (Ang II) plays a pivotal role in the exacerbation of heart failure in patients with diabetes after MI. In these patients the ICER protein levels and apoptosis in cardiomyocytes are enhanced, and p90RSK is also activated. p90RSKs are a family of serine/threonine kinases, activated by various stimuli including ischemia, reactive oxygen species, and DM. Because ICER is important in the regulation of cardiomyocyte apoptosis, we have examined whether p90RSK modulates apoptosis via regulation of the ICER protein level. First, we confirmed that Ang II activated p90RSK in cardiomyocytes. Similarly, high glucose (25 mmol/L) also activated p90RSK, whereas mannitol (25 mmol/L), which served as a hyperosmolar control, did not. Next, we investigated the involvement of p90RSK activation in cardiomyocyte apoptosis as assayed by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining. When cardiomyocytes were treated with Ang II, both ICER levels (Figure 1A and 1B) and the number of TUNEL-positive cells (Figure 1E and 1F) increased. However, these increases were inhibited in cells transduced by an adenovirus expressing dominant negative p90RSK (Ad-DN-p90RSK) (Figure 1A and 1B and Figure 1E and 1F).

In addition to overexpressing dominant negative p90RSK, we also used 1-(4-amino-7-(3-(2-methoxyethylamino)proxyl)-5-p-tolyl-7H-pyrrolo[2,3-d]pyrimidin-6-yl)-2-fluoroethanone (FMK-MEA) which is a water-soluble derivative of the previously reported fluoromethyllketone, a selective p90RSK inhibitor. Ang II-induced increase in the ICER levels and apoptosis assayed by cleaved caspase 3 expression were significantly inhibited in cardiomyocytes treated by FMK-MEA (Figure 1C and 1D), also supporting the obligatory role of p90RSK activation in these Ang II–mediated effects. We confirmed that Ang II–mediated p90RSK activation was significantly inhibited by Ad-DN-p90RSK transduction and FMK-MEA (Figure 1C and 1D), also supporting the obligatory role for p90RSK in regulating ICER levels.

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ad-DN-p90RSK</td>
<td>adenovirus expressing dominant negative form of p90RSK</td>
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<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>CA-MEK5α-Tg</td>
<td>cardiac-specific constitutively active form of MEK5α transgenic mice</td>
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<tr>
<td>CHIP</td>
<td>carboxyl terminus of Hsc70-interacting protein</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
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<tr>
<td>DM</td>
<td>diabetes mellitus</td>
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<tr>
<td>DM+MI</td>
<td>DM mice after myocardial infarction</td>
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<tr>
<td>Double-Tg</td>
<td>double transgenic mice crossing wild-type p90RSK-Tg (WT-p90RSK-Tg) and CA-MEK5α-Tg</td>
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<tr>
<td>ERK5</td>
<td>extracellular signal-related kinase 5</td>
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<tr>
<td>ERK5-CKO</td>
<td>cardiac-specific ERK5 knockout mice</td>
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<tr>
<td>ERK5-Fr</td>
<td>ERK5 fragment</td>
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<tr>
<td>FMK-MEA</td>
<td>1-(4-amino-7-(3-(2-methoxyethylamino)proxyl)-5-p-tolyl-7H-pyrrolo[2,3-d]pyrimidin-6-yl)-2-fluoroethanone</td>
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<tr>
<td>ICER</td>
<td>inducible cAMP early repressor</td>
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<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography coupled with tandem mass spectrometry</td>
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<tr>
<td>LV</td>
<td>left ventricle</td>
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<tr>
<td>LVEDd</td>
<td>left ventricular end-diastolic diameter</td>
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<tr>
<td>LVESd</td>
<td>left ventricular end-systolic diameter</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<td>NLC</td>
<td>nontransgenic littermate control</td>
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<tr>
<td>NLG-Tg</td>
<td>nontransgenic littermate control</td>
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<tr>
<td>p90RSK</td>
<td>p90 ribosomal S6 kinase</td>
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<tr>
<td>p90RSK-Tg</td>
<td>cardiac-specific wild-type p90RSK transgenic mice</td>
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<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>TL</td>
<td>tibial length</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling</td>
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<tr>
<td>Ub</td>
<td>ubiquitin</td>
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<tr>
<td>WT-p90RSK-Tg</td>
<td>cardiac-specific wild-type p90RSK transgenic mice</td>
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Activation of p90RSK Reduces CHIP Ub Ligase Activity Induced by Ang II In Vitro and After Myocardial Infarction in Diabetic Mice In Vivo

ERK5-CHIP association is critical for the upregulation of CHIP Ub ligase activity, leading to the ubiquitination and subsequent proteosomal degradation of ICER. Since p90RSK activation was crucial for an Ang II–mediated increase in ICER levels, we investigated whether p90RSK could negatively regulate CHIP Ub ligase activity and thereby stabilize the ICER protein. To detect CHIP Ub ligase activity, we performed an in vitro ubiquitination assay using a GST-ICER fusion protein as a substrate.

Next we investigated whether p90RSK activation could regulate ICER mRNA expression. As shown in Online Figure 1. Ang II induced ICER expression and apoptosis via p90RSK activation. A, Cardiomyocytes were transduced with adenovirus containing either dominant negative form of p90RSK (Ad-DN-p90RSK) or LacZ (Ad-LacZ) as a control (MOI=20) for 3 hours. They were then stimulated with Ang II (100 nmol/L and 200 nmol/L) for 24 hours. ICER, p90RSK, and tubulin were detected by Western blotting with each specific antibody. B, ICER band intensities were quantified (Fujifilm Image Gauge 4.0) and normalized relatively to tubulin band intensity. Results are expressed as fold increase in Ang II–treated compared with the untreated group. *P<0.05, compared with untreated control; mean±SD, n=3. C, Cardiomyocytes were stimulated with Ang II for 24 hours after 3 hours of FMK-MEA pretreatment, and Western blotting was performed with each specific antibody. D, ICER and cleaved caspase-3 band intensities are shown relatively to the tubulin band intensity at each point. Values are mean±SD (n=3). *P<0.05, compared with the vehicle control. E, Cardiomyocytes were transduced with Ad-DN-p90RSK or Ad-LacZ as in A and stimulated with Ang II (200 nmol/L) for 24 hours and processed for TUNEL staining. Representative pictures of TUNEL (top), α-actinin (second row), DAPI (third row), and merged (bottom) staining (×40 objective lens). Scale bars: 40 μm. F, Bar graphs showing percentage of TUNEL-positive cells (total of 400–600 cells counted). **P<0.01, *P<0.05, mean±SD, n=3.
III, A, we found that Ang II increased ICER mRNA expression. However, we could not detect any inhibition of ICER mRNA expression by Ad-DN-p90RSK transduction. These data suggested that p90RSK activation regulates ICER levels at the posttranscriptional rather than the transcriptional level.

Since CHIP Ub ligase activity requires ERK5-CHIP interaction, and since Ang II regulates this process by activating p90RSK, we examined whether Ang II–mediated p90RSK activation could regulate ERK5-CHIP interaction. In quiescent cardiomyocytes, ERK5 activity regulates the association of ERK5 with CHIP. Indeed, we found that the ERK5-CHIP

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Figure 2. p90RSK inhibited CHIP Ub ligase activity and ERK5-CHIP association. A, Ang II inhibited CHIP Ub ligase activity. Cardiomyocytes were transduced with either Ad-DN-p90RSK or Ad-LacZ for 24 hours, followed by stimulation with Ang II (200 nmol/L) for indicated times. Immunoprecipitated CHIP was subjected to an in vitro ubiquitination assay to determine CHIP Ub ligase activity using GST-fused ICER protein as a substrate (lane 1–4). The expression of Flag-tagged DN-p90RSK, CHIP, and tubulin was detected by Western blotting with each specific antibody. In vitro Ub ligase assay of GST-ICER with the Ub conjugation system and immunoprecipitates with IgG control (lane 7), and in vitro Ub ligase assay with GST only (lane 6) or without any GST substrate (lane 5) with the Ub conjugation system and immunoprecipitates with CHIP Ab (lane 1–6) are also shown. Immunoblotting with anti-CHIP (2nd from top) and anti-ICER antibody (3rd and 4th from top) after in vitro CHIP Ub ligase assay suggested that the Ub bands we observed in this assay, especially above 120 kDa, are mainly due to the ubiquitination of GST-ICER but not from GST, IgG, or endogenous CHIP as described in the method. B, Quantifications of relative CHIP Ub ligase activity from 3 independent experiments (mean±SD, n=3, *P<0.05 compared with the Ad-LacZ and untreated control). Results are expressed as the relative ratio of untreated cells in the LacZ control. C, P90RSK activation inhibited ERK5-CHIP association. Cardiomyocytes were transduced with Ad-DN-p90RSK or Ad-LacZ for 24 hours, followed by 45 minutes of Ang II stimulation. Cell lysates were immunoprecipitated with anti-CHIP and immunoblotted with anti-ERK5. Expression of ERK5, CHIP, and Flag-tagged DN-p90RSK was detected by Western blotting using each specific antibody. D, Relative coimmunoprecipitated ERK5 band intensities from 3 independent experiments were quantified (mean±SD, n=3, *P<0.05, compared with untreated control). Results are expressed as fold decrease in ERK5 band intensity in Ang II–treated cells that are expressing either Ad-LacZ or Ad-Flag-DN-p90RSK compared with untreated cells.
association was significantly decreased by stimulation of cardiomyocytes with Ang II (Figure 2C and 2D) and Ad-DN-p90RSK transduction blocked this decrease in cells treated with Ang II (Figure 2C and 2D). These results indicated a key role for p90RSK in the reduction of ERK5-CHIP association, and subsequent inhibition of CHIP Ub ligase activity in cells stimulated with Ang II.

To explore the role of p90RSK activation in the reduction of CHIP Ub ligase activity in vivo, we utilized the cardiac-specific DN-p90RSK-Tg mice. These mice showed relative resilience to cardiac ischemia/reperfusion injury,\(^3\) therefore we examined the effects of inhibiting p90RSK in diabetic mice after MI. The cardiac function of DN-p90RSK-Tg mice was unaltered,\(^3\) and the ICER levels in these transgenic were similar to that of nontransgenic littermate control (NLC) sham operated mice (Online Figure IV, A). Mice were rendered diabetic by intraperitoneal injection of STZ. On day 7 after STZ injection, both NLC and DN-p90RSK-Tg mice showed elevated random blood sugar levels with similar body weight (Online Figure V, B). We have previously shown that preoperative insulin-treatment of STZ-injected mice stabilizes cardiac function after MI, suggesting myocardial derangement in diabetic mice after MI is a function of hyperglycemia rather than a toxicity artifact of STZ.\(^3\) DN-p90RSK-Tg diabetic mice had improved survival after MI compared with that of NLC mice (Online Figure V, A). One week after MI, the LV weight/TL (tibial length) and lung weight/TL ratios were increased in NLC mice (Online Figure V, C and D), whereas these parameters were reduced in DN-p90RSK-Tg mice (Online Figure V, D). Echocardiography performed 1 week after MI showed increased left ventricular end-diastolic diameter (LVEDd) and left ventricular end-systolic diameter (LVESd) in diabetic NLC mice with a concomitant decrease in fractional shortening and ejection fraction (Online Figure V, E) compared with sham-operated non-MI controls. By contrast, we found that these MI-induced cardiac effects were attenuated in DN-p90RSK-Tg mice (Online Figure V, E).

The role of p90RSK activation in regulating CHIP Ub ligase activity, subsequent ICER levels and cardiomyocyte apoptosis in vivo were examined. As shown in Figure 3A, 3D, and Online Figure VI, CHIP Ub ligase activity was significantly decreased 1 week after MI in diabetic NLC mice but not in diabetic DN-p90RSK-Tg mice (Figure 3A and 3D and Online Figure VI). Because CHIP Ub ligase activity regulates ICER protein stability in heart failure,\(^1\)\(^2\)\(^3\) we examined the ICER protein level as well as the abundance of downstream ICER mediators, such as Bcl-2\(^1\)\(^0\) and SERCA2.\(^3\) In NLC mice, we found increased ICER levels and a concomitant reduction in Bcl-2 expression.
after MI in diabetic mice and this was rescued in DN-p90RSK-Tg diabetic mice after MI (Figure 3A, 3B, and 3D). SERCA2 expression was also reduced in NLC diabetic mice after MI. However, the reversal of this effect in DN-p90RSK diabetic mice after MI is less obvious (Figure 3B and 3D). Interestingly, the level of ERK5 phosphorylation at T218 and Y220 was decreased slightly in DN-p90RSK diabetic mice after MI (Figure 3C), suggesting a role for activated p90RSK in phosphorylating ERK5 T218/ Y220-the same residues phosphorylated by MEK5 responsible for ERK5 activation.

TUNEL-positive cells significantly increased in diabetic NLC mice after MI when compared with sham controls (Online Figure V, F and G), supporting the known role of cellular apoptosis in the development of heart failure. Notably, reduced incidence of apoptosis was found in diabetic DN-p90RSK-Tg mice after MI (Online Figure V, F and G), suggesting a functional consequence for p90RSK activation in promoting cardiac apoptosis after MI in diabetic animals.

p90RSK Activation Inhibits ERK5-CHIP Association via Competitive Binding With ERK5 and Direct Phosphorylation of ERK5-S496

On activation, ERK5 exerts its cardioprotective effect by promoting ERK5-CHIP binding, which increases CHIP Ub ligase activity. Because there is a role for p90RSK activation in promoting cardiac apoptosis after MI in diabetic mice (Figure 4A), we investigated how p90RSK regulates ERK5-CHIP association. To determine the protein domain of ERK5 responsible for the p90RSK-ERK5 and ERK5-CHIP interaction, we generated three truncated ERK5 fragments and evaluated their association with p90RSK (Figure 4A) and CHIP (Figure 4B), using a mammalian 2-hybrid assay. A plasmid containing GAL4-DBD (DNA binding domain) and the full-length p90RSK (Figure 4A) or CHIP (Figure 4B) were constructed in the pBIND vector; plasmids to express VP16-ERK5 (full-length or truncated forms) were constructed in the pACT vector. Constitutively active MEK5α (CA-MEK5α) induces forced activation of ERK5 and significantly increases ERK5-CHIP association by decreasing the inhibitory effect of the NH2-terminal kinase domain of ERK5. This ERK5-CHIP association at the helical linker domain of CHIP is fundamental for increasing CHIP Ub ligase activity. Therefore, we examined the effect of CA-MEK5α expression on the association of ERK5 and CHIP. The mammalian 2-hybrid assay demonstrated that the NH2-terminal region of ERK5 (aa1–148) and the COOH-terminal region of ERK5 (aa571–807) associate with CHIP (Figure 4A and 4B). The latter site overlaps with the p90RSK binding site.

To examine whether the wild-type p90RSK and the ERK5 fragment (ERK5-Fr, aa571–807) alter ERK5-CHIP association, we used an overexpression system. Because CHIP binding site is within the ERK5 aa571–807 region, we focused on ERK5-Fr (aa571–807). ERK5-CHIP binding was increased by CA-MEK5α (Figure 4C, lane 3). Overexpression of both wild-type p90RSK and ERK5-Fr inhibited ERK5-CHIP association (Figure 4C, lane 5–6, and Online Figure VII, A and B), suggesting that the ability of WT p90RSK to inhibit ERK5-CHIP association was due to competition between p90RSK and ERK5 for the same binding site on CHIP (Figure 8E).

Since p90RSK inhibits ERK5-CHIP association via competitive binding with ERK5, we have investigated whether Ang II–mediated p90RSK activation is required for p90RSK-ERK5 association in cardiomyocytes. Ang II increased p90RSK-ERK5 binding, and this was significantly inhibited by FMK-MEA (Figure 4D and 4E). As p90RSK activation induced by Ang II increased p90RSK-ERK5 binding, we determined the role of p90RSK activation in ERK5-CHIP binding. Wild-type p90RSK but not DN-p90RSK completely inhibited ERK5-CHIP association (Figure 4C, lane 4–5, and Online Figure VII, C and D), supporting the role of p90RSK activation in increased p90RSK-ERK5 association as well as decreased ERK5-CHIP association. Based on these results, we reasoned that because Ang II induced p90RSK but not ERK5 activation (Online Figure VIII), the regulation of ERK5-CHIP binding mediated by p90RSK activation probably occurs by a process that requires direct interaction of the 2 without ERK5 enzymatic activity being activated (Figure 8E).

Since p90RSK can associate with ERK5, we asked whether p90RSK directly phosphorylated ERK5 and as a consequence inhibited CHIP Ub ligase activity. To test this possibility, we performed an in vitro kinase assay by incubating recombinant p90RSK with GST-tagged-ERK5 fragments and found that p90RSK phosphorylated two ERK5 fragments (aa401–600 and aa601–816) (Figure 5A). In addition, LC-MS/MS analyses identified ERK5-S496 as being directly phosphorylated by p90RSK (Figure 5B and 5C). To determine whether ERK5-S496 phosphorylation is involved in the Ang II–mediated reduction of CHIP Ub ligase activity in cardiomyocytes, we generated an adenovirus expressing the ERK5-S496A mutant (Ad-ERK5-S496A). Cardiomyocytes were transduced with Ad-ERK5-S496A, Ad-ERK5-WT, or Ad-LacZ, followed by Ang II stimulation. Cell lysates were then subjected to an in vitro CHIP ubiquitination assay using a GST-ICER fusion protein as a substrate. Ang II inhibited CHIP Ub ligase activity in cardiomyocytes transduced by Ad-LacZ or Ad-ERK5-WT. In contrast, Ad-ERK5-S496A transduction abolished this inhibitory effect on CHIP Ub ligase activity (Figure 5D and 5E). In addition, the Ang II–mediated increase in ICER levels was significantly inhibited in cardiomyocytes overexpressing Ad-ERK5-S496A mutant, but not in those expressing the Ad-ERK5-WT or Ad-LacZ (Figure 5F and 5G). These findings indicate that p90RSK-mediated ERK5-S496 phosphorylation is required for inhibiting CHIP Ub ligase activity (Figure 8D and 8E).

Cardiac-Specific ERK5 Knockout Mice Showed Reduced CHIP Ub Ligase Activity After MI

To corroborate the necessity of ERK5 activation in increasing CHIP Ub ligase activity and subsequent reduction of ICER, we used ERK5 knockout mice (ERK5-CKO). Because ERK5...
activation is cardioprotective\textsuperscript{13} and ERK5-CKO mice displayed significantly increased levels of cardiac dysfunction after MI under the non-DM condition (Figure 6A, 6B and 6C), we subjected the mice to MI only. No basal cardiac abnormality was found in ERK5-CKO mice as we reported previously,\textsuperscript{34} and no increase in ICER levels was observed (Online Figure IV, B). After MI, ERK5-CKO mice showed a significant reduction in CHIP Ub ligase activity, and an increase in ICER levels (Figure 6F and 6G) compared with the NLC mice. As expected, the ERK5-CKO mouse hearts showed a significant increase in the number of TUNEL-positive cardiomyocytes compared with the NLC group (Figure 6D and 6E). These data further confirmed the importance of ERK5 in regulating CHIP Ub ligase activity and subsequent ICER levels and apoptosis in vivo (Figure 8D). Because a difference in the infarct size may correlate...
with post-MI cardiac dysfunction, we compared the permanent coronary ligation-mediated infarct size between NLC (C57BL/6) and ERK5-CKO mice. As shown in Online Figure IX we did not find any difference in the infarct size among these groups, although we found significant cardiac dysfunction and an increase in lung weight/TL in ERK5-CKO mice.

Reduction of CHIP Ub Ligase Activity and Increased ICER Levels Were Observed in WT-p90RSK-Tg Mice After MI But Were Significantly Inhibited in WT-p90RSK-Tg/Constitutive Active MEK5α Double Transgenic Mice

Thus far, we have shown the critical role of the p90RSK-ERK5 module in CHIP Ub ligase activity and ICER levels in...
Figure 6. CHIP Ub ligase activity and ICER expression after MI in ERK5-CKO mice. A, LV weight/tibial length (TL), and B, lung weight/TL after MI in NLC and ERK5-CKO mice as indicated (*P<0.05, mean±SD, n=8–9). C, Echocardiographic data obtained after MI in NLC and ERK5-CKO mice as indicated. FS indicates fractional shortening; EF, ejection fraction (**P<0.01, mean±SD, n=8–9). D, Cardiomyocyte apoptosis in remote areas was enhanced in ERK5-CKO mice. Animals were subjected to either MI or sham. TUNEL-positive cardiomyocytes were counted among >8000 to 10 000 nuclei in the remote area of each mouse heart after 1 week of surgery (×40 objective lens). Scale bars, 40 μm. Representative pictures of TUNEL (top), 4′, 6′-diamidino-2-phenylindole (DAPI) (middle), and α-actinin merged with TUNEL and DAPI staining (bottom) of the remote area from NLC and ERK5-CKO mice subjected to MI or sham operation as indicated. E, Bar graph showing TUNEL-positive cells (%) in various animals (*P<0.05, mean±SD, n=3–4). F, Heart samples from sham and MI groups in NLC mice and in ERK5-CKO mice were collected and CHIP Ub ligase activity assay was performed as described in methods (top). CHIP, ERK5, and tubulin expressions were detected by Western blot. G, Relative CHIP Ub ligase activity (left, n=9) and ICER expression (right, n=3) were quantified as described in Figure 2B and Figure 1B, respectively (**P<0.01, *P<0.05, mean±SD).
Figure 7. ERK5 activation prevented the exacerbation of LV dysfunction after MI in WT-p90RSK-Tg mice. A, Kaplan-Meier survival analysis in NLC, WT-p90RSK-Tg, and WT-p90RSK-Tg/CA-MEK5a-Tg (Double-Tg) after MI. Survival rate in NLC (n = 29), WT-p90RSK-Tg (n = 36), and Double-Tg mice (n = 27) after MI are plotted. Overall survival was significantly higher in Double-Tg compared with WT-p90RSK-Tg mice. *P < 0.05 compared with WT-p90RSK-Tg group. B, Body weight 1 week after MI in NLC, WT-p90RSK-Tg, and Double-Tg mice (mean ± SD, n = 17–19). C and D, LV weight/TL (C) and lung weight/TL (D) after MI in NLC, WT-p90RSK-Tg, and Double-Tg mice (*P < 0.05, **P < 0.01, mean ± SD, n = 17–19). E, Echocardiographic data obtained after MI in NLC, WT-p90RSK-Tg, and Double-Tg mice. LVEDd indicates left ventricular end-diastolic dimension; LVESd, left ventricular endsystolic dimension; TL, tibial length; FS, fractional shortening; and EF, ejection fraction (*P < 0.05, **P < 0.01, mean ± SD, n = 17–19). F, Cardiomyocyte apoptosis in the remote area was increased in WT-p90RSK-Tg mouse hearts, which was inhibited in Double-Tg mice. TUNEL-positive cardiomyocytes were counted in the remote area of each mouse heart as described in Figure 6D. Representative pictures of TUNEL (top), DAPI (middle), and α-actinin merged with TUNEL and DAPI staining (bottom) of the remote area from NLC, WT-p90RSK-Tg, and Double-Tg mice subjected to MI or sham operation (×40 objective lens). Scale bars, 40 μm. G, Bar graph showing TUNEL-positive cells (%) in various animals (*P < 0.01, **P < 0.05, mean ± SD, n = 3).
Figure 8. CHIP Ub ligase activity and ICER expression after MI in NLC, WT-p90RSK-Tg, and Double-Tg mice. A through C, Heart samples from control and MI groups in NLC, WT-p90RSK-Tg, and Double-Tg mice were collected and CHIP Ub ligase activity assay was performed as described in Methods (top). Western blot shows A, CHIP, ICER, p90RSK, and ERK5; and B, Bcl-2, SERCA2, and tubulin expression. C, Quantifications of relative CHIP Ub ligase activity and ICER expression (upper), Bcl-2 and SERCA2 expression (lower) as described in Figure 2B and Figure 1B (**P<0.01, *P<0.05, mean±SD, n=3).

D, Model of DM or Ang II-mediated p90RSK-ERK5-CHIP signal transduction pathway that regulates cardiac apoptosis and subsequent cardiac dysfunction.

E, Scheme depicting p90RSK-mediated regulation of the ERK5-ChIP module. At the basal level, inactive p90RSK inhibits the D-domain to bind with ERK5. p90RSK-free ERK5 associates with CHIP at its linker and U-box domain and maintains its CHIP Ub ligase activity to prevent ICER induction and subsequent apoptosis. However, once p90RSK is activated, the inhibition of the kinase domain is released and the D-domain of p90RSK associates with the ERK5 COOH-terminal domain, leading to compete with ERK5-ChIP association and ERK5-S496 phosphorylation, which disrupts ERK5-ChIP interaction. The disruption of ERK5-ChIP interaction inhibits CHIP Ub ligase activity, increases ICER induction, and induces apoptosis.
vitro, but the role of this module in vivo remains unclear. To clarify the function of the p90RSK-ERK5 signaling cascade in vivo, we crossed WT-p90RSK-Tg mice with those expressing constitutive active MEK5α (CA-MEK5α-Tg) to generate double transgenic mice (Double-Tg). Cardiac function is normal in CA-MEK5α-Tg and WT-p90RSK-Tg mice at 8 to 12 weeks of age as we previously described.\(^{35,36}\) Double-Tg also shows normal cardiac function (fractional shortening % = 67.7±3.7, n=3). No increase in ICER levels was observed in WT-p90RSK-Tg or Double-Tg mice (Online Figure IV, A).

Nondiabetic mice were subjected to a simple MI. After MI, the mortality of WT-p90RSK-Tg mice was significantly greater than NLC mice. The mortality of Double-Tg was similar to NLC mice (Figure 7A). There was no difference in body weight among the 3 groups (Figure 7B). One week after MI, the LV weight/TL and lung weight/TL ratios were higher in WT-p90RSK-Tg mice compared with NLC mice (Figure 7C and 7D) but lower in Double-Tg mice compared with WT-p90RSK-Tg mice (Figure 7C and 7D). Myocardial contractile function as assessed by echocardiography was diminished in WT-p90RSK-Tg mice compared with NLC after MI (Figure 7E). Myocardial contractile function as assessed by echocardiography was preserved in Double-Tg mice (Figure 7E). We previously reported that CA-MEK5α-Tg mice develop contractile dysfunction 1 week after simple MI.\(^{31}\) In contrast, we found that activation of ERK5 by CA-MEK5α could reverse the detrimental effect of p90RSK activation on cardiac function after a simple MI, consistent with the idea that p90RSK activation accelerates cardiac dysfunction and heart failure after MI, possibly via inhibiting ERK5 activity. We also assessed the infarct size after permanent coronary ligation in NLC (FVB) mice, in diabetic mice, and in Double-Tg mice. As shown in Online Figure IX we found no difference in infarct size among these 3 groups. However, we noted significant cardiac dysfunction and increase of lung weight/TL in diabetic mice after MI. These data suggest that the major difference in cardiac dysfunction observed after one week of MI stems from cardiac remodeling in the surrounding, noninfarcted area rather than simply from infarct expansion.

Next, we investigated the involvement of CHIP Ub ligase activity in regulating ICER levels. Compared with the sham NLC mice, WT-p90RSK-Tg mice have reduced CHIP Ub ligase activity 1 week after MI. In contrast, no decrease in CHIP Ub ligase activity was observed in Double-Tg mice (Figure 8A and 8C). The increase in ICER level and concomitant reduction in Bcl-2 expression in WT-p90RSK-Tg mice were also detected, and these changes were mostly reversed in Double-Tg mice (Figure 8A, 8B, and 8C). Similar to NLC diabetic mice after MI (Figure 3B and 3D), SERCA2 expression was also reduced in WT-p90RSK-Tg mice after MI (Figure 8B and 8C). However, restoration of SERCA2 expression in Double-Tg mice after MI was observed (Figure 8B and 8C), contrasting what was observed DN-p90RSK diabetic mice after MI (Figure 3B and 3D).

Finally, WT-p90RSK-Tg mice have increased apoptosis one week after MI compared with sham NLC mice, and this phenomenon was reversed in Double-Tg mice (Figure 7F and 7G). Taken together, ERK5 activation suppresses p90RSK-mediated cardiac apoptosis after MI in vivo.

Discussion

In this study, we demonstrated a critical role for p90RSK activation in the reduction of CHIP Ub ligase activity and subsequent increase in the ICER protein levels (Figure 8D and 8E). p90RSK activation promotes its association with ERK5 and phosphorylates it at S496. This phosphorylation appears to be a necessary and sufficient step in the ability of p90RSK to promote the dissociation of the ERK5-CHIP complex, which leads to reduced CHIP Ub ligase activity. The physiological significance of p90RSK activation in diabetic mice after MI lies in its ability to decrease CHIP Ub ligase activity. This mechanism is reversed in diabetic DN-p90RSK-Tg mice, which show increased CHIP Ub ligase activity and recovery of cardiac function.

Depletion of ERK5 inhibited CHIP Ub ligase activity and enhanced ICER levels after MI, suggesting a causal role of ERK5 in controlling CHIP Ub ligase activity and subsequent ICER stability. Supporting the role of ERK5 as a negative regulator of p90RSK-mediated CHIP Ub ligase inhibition, we demonstrate that diabetic WT-p90RSK-Tg mice exhibit diminished CHIP Ub ligase activity after MI, but that coexpressed CA-MEK5α in Double-Tg mice leads to restoration of CHIP Ub ligase activity, and prevents cardiac dysfunction, at least in part by preventing myocardial apoptosis.

Our studies focus on defining the molecular mechanism of cardiac dysfunction after MI, which is a prevalent but poorly understood consequence of diabetes. Therefore, we established a diabetic model of MI in which post-MI biochemical and physiological sequelae could be carefully examined in the immediate post-MI period.\(^{31}\) We observed that p90RSK activation was significantly increased in DM. Consistent with our previous work in WT-p90RSK-Tg mice, we see cardiac dysfunction and significant fibrosis after 8 months of age—a phenotype similar to that seen in diabetic cardiomyopathy.\(^{36}\) Furthermore, we found that DN-p90RSK-Tg mice do not have the same degree of cardiac dysfunction after MI in a diabetic model, supporting an important role for p90RSK in the development of cardiac dysfunction after MI in diabetes. The ability of p90RSK activation to decrease functionality of the ERK5-CHIP module suggests that cardiac-specific ERK5 knockout mice (ERK5-CKO) mimic the deleterious effect of diabetes on the heart. Therefore, we modeled MI only in WT-p90RSK-Tg (Figure 8) and ERK5-CKO mice (Figure 6). As mentioned above, we found that CHIP Ub ligase activity after MI was inhibited in ERK5-CKO mice compared with NLC mice. We also observed that CHIP activity in the sham operation group is much lower in C57BL/6 than FVB mice. The background of ERK5-CKO mice is C57BL/6, whereas the background of DN-p90RSK-Tg, WT-p90RSK-Tg, and Double-Tg is FVB. Therefore, this may explain why C57BL/6 mice are much more sensitive to MI than FVB strain and show much
higher mortality after MI surgery. Further investigation will be necessary to clarify this issue.

Ranganathan et al. reported that the COOH-terminal domain of the D motif of p90RSK is an ERK5 binding site and that p90RSK activation was required for full association between p90RSK and ERK5. Consistent with this report, in our present study, we found that p90RSK activation is a key event in disrupting ERK5-CHIP interaction by (1) competitive binding of p90RSK to ERK5 causing displacement of CHIP from ERK5 and (2) phosphorylation of ERK5 at S496. Forced activation of ERK5 induced by insulin growth factor-1 or CA-MEK5α inhibits the association of p90RSK with ERK5. In addition, activated ERK5 significantly increases ERK5-CHIP association by relieving the inhibitory effect of the NH2-terminal kinase domain of ERK5 (Figure 8D and 8E). ERK5-CHIP association at the helical linker domain of CHIP is critical for increasing CHIP Ub ligase activity.13 In support of this, the elevation of ERK5 activity in Double-Tg mice reversed the inhibitory effect of p90RSK activation on CHIP Ub ligase activity. Taken together, our data demonstrate that p90RSK activation influences the ability of its own COOH-terminus docking domain to bind ERK5 and phosphorylate residue S496. The p90RSK-ERK5 interaction and ERK5-S496 phosphorylation disrupt ERK5-CHIP association and decrease CHIP Ub ligase activity (Figure 8D and 8E). It is possible that the association between p90RSK and ERK5 is necessary for p90RSK to phosphorylate S496 ERK5, or that p90RSK-mediated S496 phosphorylation of ERK5 leads to dissociation of p90RSK and ERK5. To determine which of these possibilities is true will require further investigation.

Our data show that p90RSK activation regulates ICER protein stability by regulation of CHIP Ub ligase activity. In support of this, (1) an ERK5-S496A mutant significantly inhibits Ang II–mediated reduction of CHIP Ub ligase activity and subsequent increase in ICER levels (Figure 5), (2) CHIP is critical for ERK5-mediated decrease in ICER levels,13 and (3) depletion of CHIP increases ICER stability.13 CHIP activity can regulate a variety of molecules including FOXO1,38 ASK1,39 and p53,40,41 and we cannot exclude the possibility that p90RSK can regulate the expression of these molecules via CHIP, and that dysregulation of these molecules contribute to cardiac dysfunction. In addition, p90RSK and ERK5 can also regulate proteins with CHIP and ICER-independent mechanism including Na+/?H+ exchanger,19 renin-angiotensin system,36,42 and PPARs activities.43 Further investigation is necessary to clarify the role of these molecules in p90RSK/ERK5-mediated cardiac dysfunction, especially in DM, both in vitro and in vivo.

Naito et al reported that CHIP expression was decreased with concomitant upregulation of p53 expression within four days after MI.41 In our study, there was no significant decrease in CHIP expression or increase in p53 expression at 7 days after MI in diabetic mice (data not shown). Instead, we found a significant decrease in CHIP Ub ligase activity in our diabetic mice after MI. The regulatory mechanism responsible for the downregulation of CHIP expression was not identified in the previous studies.41 Our present study suggests a role for p90RSK activation in regulating ERK5-CHIP association and subsequent CHIP Ub ligase activation. Whether through an early decrease in CHIP expression or a later decrease in its Ub ligase activity, the net effect is promotion of the proapoptotic milieu contributing to poor functional recovery in diabetic mice after MI. Based on our results on the role of p90RSK-ERK5-CHIP described in our current work, ERK5 activation may reverse the decrease in Ub ligase activity and ameliorate cardiac dysfunction after MI in DM hearts.

Last, we close by commenting on the protective function of ERK5 after MI with or without superimposed DM. In this study, we showed that cardioprotective effect of ERK5 activation is particularly significant after MI in the context of DM. Previously, we reported that the cardiac function did not improve after 1 week of MI in the absence of DM in CA-MEK5α-Tg mice compared with NLC mice.31 These data suggest that ERK5 activation does not have a significant cardioprotective role in our MI model 1 week after permanent ligation. Although the role of ERK5 activation in cardiac remodeling after permanent ligation is limited, we found that cardiac protection was enhanced in hyperglycemic CA-MEK5α-Tg mice,31 suggesting that ERK5 activation has a significant role in DM after MI rather than being a consequence of MI per se, at least during the acute phase (within 1 week) after MI.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Diabetes mellitus (DM) is an independent risk factor for both mortality and morbidity after myocardial infarction (MI). Induction of inducible cAMP early repressor (ICER), a proapoptotic transcriptional repressor, accelerates myocyte apoptosis and subsequent cardiac dysfunction in DM mice after MI.
- Activation of ERK5 positively regulates chaperone-dependent E3 ubiquitin (Ub) ligase CHIP (carboxyl terminus of Hsp70-interacting protein)-mediated ICER ubiquitination and subsequent protein degradation, which protects cardiomyocytes from apoptosis.

What New Information Does This Article Contribute?

- ERK5-CHIP module is one of the major targets of p90RSK in diabetic hearts.
- Depletion of ERK5 inhibits CHIP Ub ligase activity and increases ICER levels after MI.
- Diabetic wild-type p90RSK transgenic mice exhibit diminished CHIP Ub ligase activity after MI. However, when these mice coexpress a constitutively active form of MEK5Δ in double transgenic mice, CHIP Ub ligase activity is restored, also restoring cardiac dysfunction, at least in part by preventing myocardial apoptosis.
- The activation of p90RSK abrogates ERK5-mediated CHIP Ub ligase activation, and accelerates apoptosis and cardiac dysfunction in the DM + MI condition.

DM reduces survival after MI primarily due to acceleration of heart failure. We have found that the activation of p90RSK abrogates ERK5-mediated CHIP Ub ligase activation, and accelerates apoptosis and cardiac dysfunction in the DM + MI condition. Our data demonstrate that p90RSK activation influences the ability of its own COOH-terminus docking domain to bind ERK5 and phosphorylate residue S496. The p90RSK-ERK5 interaction and ERK5-S496 phosphorylation disrupt ERK5-CHIP association and decrease CHIP Ub ligase activity. The regulatory mechanism responsible for the downregulation of CHIP expression after MI has not yet been identified. Our present study suggests a role for p90RSK activation in regulating ERK5-CHIP association and subsequent CHIP Ub ligase activation, either through an early decrease in CHIP expression or a later decrease in its Ub ligase activity. The net effect is promotion of the proapoptotic milieu contributing to poor functional recovery in diabetic mice after MI. ERK5 activation may reverse the decrease in Ub ligase activity and ameliorate cardiac dysfunction after MI in DM hearts. Understanding the role and molecular mechanisms how p90RSK targets ERK5-CHIP ubiquitin E3 ligase activity should provide insights into mechanisms contributing to poor cardiac recovery after MI in DM and possibly reveal a novel therapeutic target.
p90RSK Targets the ERK5-CHIP Ubiquitin E3 Ligase Activity in Diabetic Hearts and Promotes Cardiac Apoptosis and Dysfunction
Nhat-Tu Le, Yuichiro Takei, Tetsuro Shishido, Chang-Hoon Woo, Eugene Chang, Kyung-Sun Heo, Hakjoo Lee, Yan Lu, Craig Morrell, Masayoshi Oikawa, Carolyn McClain, Xin Wang, Cathy Tournier, Carlos A. Molina, Jack Taunton, Chen Yan, Keigi Fujiwara, Cam Patterson, Jay Yang and Jun-ichi Abe

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Online figure I. p90RSK activation in cardiomyocytes (A-D) Cardiomyocytes were stimulated with Ang II (200 nM) (A and B) or high glucose or mannitol (C and D) for the indicated times. p-p90RSK, p90RSK and tubulin were detected by Western blotting of total cell lysates with each specific antibody. (B and D) Intensities of p-p90RSK protein bands were quantified using a Fujifilm image-analysis program (Image Gauge 4.0) and were calculated relatively to the intensity of the tubulin band at each time point. Results were expressed as fold increase of p-p90RSK in AngII-treated cells compared to untreated cells. Shown is mean ± S.D. (n = 3). ** p < 0.01, * p < 0.05 compared to untreated cells.
Online figure II. Ad-DN-p90RSK and FMK-MEA, a specific p90RSK inhibitor, blocked Ang II-mediated p90RSK activation in cardiomyocytes (A) Cardiomyocytes were stimulated with Ang II (200 nM), and p90RSK kinase activity was detected using an in vitro kinase assay as described in methods. The p90RSK kinase activity (upper) was normalized to its expression level, which was evaluated by Western blotting of p90RSK (lower). The blots are representative of data obtained from three separate experiments. (B) Cardiomyocytes were pretreated with vehicle or FMK-MEA for 3 hrs and stimulated with Ang II (200 nM) for the indicated times. p-p90RSK, p90RSK and tubulin were detected by Western blotting of total cell lysates with each specific antibody. The blots are representative of data obtained from three separate experiments.

A

B
Online figure III. Ad-DN-p90RSK did not inhibit Ang II-mediated ICER mRNA expression in cardiomyocytes (A), but Ad-WT-p90RSK enhanced ICER protein levels (B). (A) Cardiomyocytes were transduced with Ad-LacZ or Ad-DN-p90RSK for 24 hrs. Cells were then stimulated with Ang II (200 nM) for 30 min, and ICER mRNA level was detected by qRT-PCR as described in methods. (B) Cardiomyocytes were transduced with Ad-LacZ or Ad-WT-p90RSK for 24hrs. ICER, p90RSK and tubulin were detected by Western blotting using the total cell lysates with each specific antibody (left). Intensities of ICER protein bands were quantified using a Fujifilm image-analysis program (Image Gauge 4.0) and were calculated relatively to the intensity of the tubulin band at each time point. Results were expressed as fold increase of ICER in Ad-WT-p90RSK-transduced cells compared to the Ad-LacZ-transduced cells. Shown is mean ± S.D. (n = 3). * p < 0.05.
Online figure IV. DN-p90RSK-Tg, WT-p90RSK-Tg, Double-Tg, and ERK5-CKO mice showed no difference of ICER expression in sham operated mice. Expression of ICER and tubulin in heart samples collected from sham and MI in NLC, sham in DN-p90RSK-Tg, WT-p90RSK-Tg and Double-Tg mice (A), and heart samples from sham in NLC and ERK5-CKO (B).
Online figure V. Inhibition of p90RSK activation prevented the exacerbation of LV dysfunction after MI in diabetic mice. (A) Kaplan-Meier survival analysis in diabetic NLC (n=37) and DN-p90RSK-Tg (n=15) after MI. Overall survival was significantly higher in DN-p90RSK-Tg compared to NLC mice. * p < 0.05 compared to NLC group. (B) Random blood sugar (BS) and body weight at one week after STZ injection for coronary ligation (DM + MI) or vehicle-treated sham operation groups in NLC or DN-p90RSK-Tg mice. (mean ± S.D., n = 9-10) (C) LV weight/TL (mean ± S.D., n = 9-10) and (D) lung weight/TL in DM + MI or vehicle-treated sham operation groups in NLC or DN-p90RSK-Tg mice one week after surgery. (**p <0.01, mean ± S.D., n=9-10). (E) Echocardiographic data obtained from DM + MI, or vehicle treated sham operation groups in NLC or DN-p90RSK-Tg mice one week after surgery. LVEDd, left ventricular end-diastolic dimension; LVESd, left ventricular end-systolic dimension; TL, Tibial length. (*p<0.05, **p <0.01, mean ± S.D., n=9-10). (F) Cardiomyocytes apoptosis in the remote area was increased in DM + MI group, which was inhibited in DN-p90RSK-Tg mice. TUNEL-positive cardiomyocytes were counted in the remote area as described in Fig.6D. Representative pictures of TUNEL (top), DAPI (middle), and merged of α-actinin with TUNEL and DAPI staining (bottom) of the remote area from NLC mice subjected to sham or DM+MI, and DN-p90RSK-Tg mice subjected to DM + MI operation. 40X objective lens. Scale bars: 40 µm (G) A bar graph showing TUNEL-positive cells (%) in NLC and DN-p90RSK-Tg. (* p <0.05, mean ± S.D., n=3).
Online figure VI. Quantification of CHIP ubiquitin E3 ligase activity. The activities of CHIP Ub E3 ligase were assessed by quantifying ubiquitinated GST-ICER fusion proteins. Briefly, protein was extracted by modified RIPA buffer, and then immunoprecipitated with anti-CHIP antibody. CHIP proteins were immunoprecipitated by protein A and G agarose mixture. After that, GST-ICER protein as added to each sample. CHIP Ub E3 ligase activity was detected using Ubiquitin-Protein Conjugation kit. Science Lab 2001 Image gauge software (version 4; Fuji Photo Film, Tokyo, Japan) was used for the analysis. Ubiquitinated GST-ICER expressions were calculated in equal area described in white broken squares. Relative levels were indicated as based on means of NLC sham mice.
Online figure VII. p90RSK kinase activation inhibits ERK5-CHIP association. (A) Both wild type p90RSK (WT-p90RSK) and ERK5-Fr (aa571-807) disrupted ERK5-CHIP association. After plasmids were transfected as indicated, cell lysates will be immunoprecipitated with anti-ERK5 and then immunoblotted with anti-CIP. Protein expression was determined by immunoblotting with each specific antibody. (B) Relative ERK5-CHIP binding was quantified as described in Fig.2D (mean±S.D., n=3; (**)p<0.01, * p<0.05 compared to both Myc-CIP and CA-MEK5α transfected cells (the third black bar from left). Results are expressed as fold decrease in ERK5-CHIP binding in WT-p90RSK (grey bars) and ERK5-Fr (aa581-807) (white bars) transfected cells compared to the control cells (the third black bar from left). (C) WT-p90RSK but not DN-p90RSK fully disrupted ERK5-CHIP interaction. ERK5-CHIP binding and each protein expression were detected as described in (A). (D) Relative ERK5-CHIP binding was quantified as described in Fig.2D. Results are expressed as fold decrease in ERK5-CHIP binding in cells over-expressing WT-p90RSK. **p<0.01, mean±S.D., n=3.
Online figure VIII. Ang II increased only p90RSK but not ERK5 kinase activity in cardiomyocyte. Cardiomyocytes were stimulated with Ang II (200 nM) or H$_2$O$_2$ (200 µM) for 15 min. p-p90RSK, p90RSK, p-ERK5, ERK5, p-ERK1/2, and tubulin were detected by Western blotting using the total cell lysates with each specific antibody. Representative immunoblots from duplicate experiments are shown.
Online figure IX. Evaluation of infarction size at one week after MI surgery.

(A) Representative pictures of LV tissue sections by Masson’s trichrome staining. Infarct areas were described in black triangle between broken lines. 4X objective lens. Scale bars: 1000 µm. (B) Comparision of MI sizes. The sizes were not significantly changed in each groups. Data are shown as means ± S.D. (n=3).
Expanded materials and methods:

**Animals:** The *erk5-flox* mice were previously described (ERK5^flox/flox)^1. ERK5^flox/flox mice on a C57bl/6 background were mated with mice expressing Cre under cardiac α-myosin heavy chain (α-MHC) promoter to generate cardiac-specific ERK5 knockout mice (ERK5-CKO)^2. The α-MHC-Cre mice on C57bl/6 background were kindly provided by Dr. E. Dale Abel^3. Rat wild type and dominant negative mutant (K94A/K447A) p90RSK1 cDNA was subcloned into a pBluescript-based Tg vector between the 5.5-kb murine-α-MHC promoter and 250-bp SV-40 polyadenylation sequences (a kind gift from Dr. J. Robbins, Children’s Hospital Research Foundation, Cincinnati, Ohio) as we have previously described^4. The mouse constitutively active form of MEK5α (CA-MEK5α, S311D/T315D) cDNA was subcloned into a pBluescript-based Tg vector between the 5.5-kb murine-α-MHC promoter and 250-bp SV-40 polyadenylation sequences, and we generated three different lines of CA-MEK5α-Tg in FVB strains. All three lines showed a similar phenotype and MEK5α expression as we reported previously^5. The animal studies were performed in accordance with the University Committee on Animal Resources and institutional guidelines at University of Rochester Medical Center.

**Antibodies:** Antibodies were purchased from the following vendors: anti-p90RSK1 (C-21, #SC-231), anti-Ub (P4D1, # SC-8017), anti-HA (Y-11, SC-805), anti-CHIP (H-231, SC-66830), and anti-VP16 (#SC-7546) from Santa Cruz (Santa Cruz Biotechnology, CA); anti-phospho-p90RSK (Ser380, # 9341), anti-phospho-ERK5 (Thr218/Tyr220, #3371L), anti-ERK5 (#3372), anti-cleaved caspase 3 (# 9664), and anti-Bcl-2 (#2876) from Cell Signaling (Cell Signaling Technology Inc, Danvers, MA); anti-tubulin (T-5168) and anti-Flag (# F-3165) from Sigma (Sigma, St. Louis, MO); anti-ICER antibody was generated as previously described^6.

**Reagents:** Angiotensin II was purchased from Calbiochem (Cat # 05-23-0101, an Affiliate of Merck KGaA Darmstadt, Germany). D-glucose (Dextrose) and Mannitol was from Sigma (St. Louis, MO). Hydrogen Peroxide was purchased from Sigma (#216763). GST fused active recombinant full-length rat RSK1 (Cat # 14-479, Lot # 31236AU) was purchased from Millipore (Millipore, Billerica, MA).

**Plasmid and adenovirus vector construction:** Mouse ERK5 (mERK5α) and a constitutively active form of MEK5α (CA-MEK5α) were cloned as previously described^7. Both the VP16 full-length wild type and truncated forms of mERK5α in the pACT vectors were generated by inserting the mouse full-length wild type and truncated forms of mERK5α isolated from pcDNA3.1-mERK5α into BamHI and NotI sites of the pACT vector. Glutathione S-transferase (GST)-ICER was created by ligation of fragment from BamHI-EcoRI digest of pCMVtag2b-ICER into the same sites in the pGEX-KG vector (Amersham Biosciences, GE Healthcare Biosciences, Pittsburgh, PA). ERK5 S496A point mutations were created from the mERK5α full-length using the QuikChange site-directed mutagenesis kit (Stratagene). For adenovirus preparation, DN-p90RSK construct was cloned into an AdEasy-CMV system (QBIOfGen, Carlsbad, CA) with SalI and HindIII restriction enzymes^8. All constructs were verified by DNA sequencing. Adenovirus vector containing β-galactosidase (Ad-LacZ) was used as a control virus.

**Cell culture:** Primary cultures of neonatal rat ventricular cardiomyocytes were performed as described previously^9,10. Briefly, neonatal rat ventricular cardiomyocytes were obtained by enzymatic digestion of cardiac ventricles from 2 -3 day old Sprague-Dawley rat neonates. The ventricular tissues were subjected to multiple rounds of enzymatic digestion using collagenase II (1.2 mg/ml; Worthington Biochemicals Corp., Lakewood, NJ, Cat # 4177). Cardiomyocytes were collected after every two rounds of collagenase digestion by centrifugation at 800 rpm for 5 min at 4 ºC. Non-myocytes were removed by pre-plating on culture dishes. Cardiomyocytes were then plated on pre-coating gelatin plates, in DMEM low glucose supplemented with 10% fetal bovine serum (FBS) and 10 µM cytosine 1-β-D-arabinofuranoside (Sigma). Addition of cytosine 1-β-D-arabinofuranoside can inhibit the growth of contaminating non-myocytes.
More than 90% of cells were cardiomyocytes (positive for α-actinin). Next days, cardiomyocytes were washed thoroughly with PBS to remove any unbound cells, then maintained for another 3 days to let the cells stable before using for experiments. HeLa cells were maintained in DMEM (GIBCO, Carlsbad, CA) supplemented with 10% FBS, 50 units/mL penicillin, and 50 µg/mL streptomycin.

**ICER mRNA detection:** Rat neonatal cardiomyocytes cultured on 6-well-plates pre-coated with gelatin at ~ 80% density. After 24 hours, cells were washed thoroughly with PBS. Three days later, cells were infected with either Ad-LacZ as a control (MOI=20) or Ad-DN-p90RSK (MOI=20) overnight, followed by stimulation with Ang II (200 nM) for 30 min. Total RNA was then isolated using RNeasy Plus Mini Kit (Cat. # 74134, QIAGEN Sciences, Maryland 20874, US) according to the manufacturer’s instruction. Genomic DNA was eliminated by gDNA Eliminator column, which is included in the Kit. Single strand cDNA synthesis was done in 50 µL reaction volume containing 2 µg of purified RNA, 5 µL 10X buffer, 11 µL MgCl2, 10 µL dNTPs, 2.5 µL random hexamer, 1.25 µL oligo dT, 1 µL RNAse inhibitor, and 0.75 µL reverse transcriptase enzyme (TaqMan Reverse Transcription Reagents, N808-0234, made for Applied Biosystems by Roche Molecular systems, Inc., Branchburg, New Jersey, US). Target cDNA levels were quantified by real-time RT-PCR using a MyiQ™2 Optics Module (Bio-Rad Laboratories, Inc.). Each reaction mixture (20 µL) contained cDNA synthesized from 20 ng of total RNA, 10 µL of iQ™SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 0.5 µmol/L of each primer. The real-time PCR protocol consisted of an initial step at 95°C for 3 min, followed by 40 cycles: 95°C for 10 sec and annealing at 55°C for 30 sec. The Δ∆Ct method was used to calculate the fold change in mRNA expression: ΔCt = Ct (target gene) – Ct (housekeeping gene), ΔΔCt = ΔCt (treatment) - ΔCt (control), fold change = 2 (-ΔΔCt). Primer sequences are as follows: rat ICER forward: 5’-ATG GCT GTA ACT GGA GAT GAA ACT G -3’ and reverse: 5’-CAC CTT GTG GCA AAG CAG TA-3’; Rat β-Tubulin forward: 5’-GGA GGA TGC TGC CAA TAA CT-3’ and reverse: 5’-GGT GGT GAG GAT GGA ATT GT-3’. 11.

**Mammalian two-hybrid analysis:** The association between ERK5 and CHIP was analyzed using a CheckMate mammalian two-hybrid system (Promega Corporation). A pBIND vector containing Gal4-DBD was fused with the full-length of CHIP. pACT vector containing VP16 was fused with either full-length or truncated forms of ERK5. Sub-confluence HeLa cells plated in 12-well-plates were transfected using Opti-MEM (Invitrogen, Carlsbad, CA) containing Plus-Lipofectamine mixture with PG5-Luc vector and various pBIND and pACT plasmids in the presence or absence of pHA-CA-MEK5α, as indicated, for 4 hrs. Cells were then washed and fresh DMEM medium supplemented with 10% FBS was added. The pG5-Luc vector contains five Gal4 binding sites upstream of a minima TATA box, which in turn, is upstream of the firefly luciferase gene. Since pBIND also contains the Renilla luciferase gene, the expression and transfection efficiencies were normalized to the Renilla luciferase activity. The association between ERK5 and p90RSK was analyzed using the same method as for analysis ERK5-CHIP association, using pBIND vector contains Gal4-DBD was fused with the full-length p90RSK. Cells were collected 24 hours after transfection unless otherwise stated, and the luciferase activity was assayed with the dual luciferase reporter system (Promega, Madison, WI, USA) using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). Transfections were performed in triplicate, and each experiment was repeated at least thrice.

**Immunoprecipitation and Western blot analysis:** Cells were washed 3 times with phosphate-buffered saline (PBS), and cell extracts were prepared in modified radioimmunoprecipitation assay 1 (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1:200-diluted protease inhibitor cocktail (Sigma, MO, USA), 1 mM PMSF and 10 mM NEM). Mouse hearts were washed 3 times with cold Hanks’ Balanced Salt Solution (HBSS) (Mediatech. Inc, Manassas, VA 20109). After the hearts was frozen in liquid nitrogen, the hearts were ground with mortar and pestle into powder. This heart powder was homogenized in 1 mL of modified RIPA buffer, followed by sonication (5 x 10 second pulse) and then centrifuged at 14,000g (4°C for 30 min), and...
protein concentration was determined as previously described. Immunoprecipitation with anti-ERK5 antibody or anti-CHIP antibody was performed as described previously. Bound proteins were released in 2x SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, transferred onto a Hybond enhanced chemiluminescence nitrocellulose membrane, and visualized by using the Enhanced Chemiluminescence Substrate (PerkinElmer, Shelton, CT, USA) according to the manufacturer's instructions. Immunoblotting was performed as previously described.

**p90RSK in vitro kinase assay:** p90RSK activity was measured by autophosphorylation and GST-ERK5 phosphorylation as described previously. GST-ERK5 wild-type and mutants (1 µg) were resuspended in 50 µL of kinase assay solution containing 10 mM MgCl₂, 10 mM MnCl₂, and 25 mM HEPES (pH 7.4), and the kinase reaction was initiated by adding 1 µg active RSK1 (Rsk1/MAPKAP Kinase 1a, active, Cat # 14-479, Millipore), 15 µM ATP (Cat # 10519979001, Roche Applied Science, Indianapolis, IN), and 3 µCi [γ-32P]ATP. The reaction at 30°C was for 30 min and terminated by adding Laemmli’s sample buffer. Auto-phosphorylation and GST-ERK5 phosphorylation were determined by densitometry at the correct molecular weights in the linear range of film exposure with the use of a scanner and NIH Image J.

**In vitro ubiquitination assay with GST-ICER:** Lysates of neonatal rat ventricular myocytes or ground frozen heart tissues were prepared in modified radioimmunoprecipitation assay (RIPA) butter [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Nonidet P-40; 0.1% sodium dodecyl sulfate (SDS); 1:200-diluted protease inhibitor cocktail ( Sigma, St. Louis, MO); 1 mM PMSF; and 10 mM NEM]. Lysates were then immunoprecipitated using an anti-CHIP antibody and incubated overnight at 4°C with gentle shaking. After that, 30 µL of the 50% protein A and G agarose mixture (1:1 volume ratio) (protein A agarose - Cat # 15918-014, Invitrogen; protein G agarose - Cat # 15920-010, Invitrogen) was added and incubated for another 2 hrs, with gentle shaking. The immunoprecipitated CHIP was then washed five times using washing buffer [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% Nonidet P-40; 0.1% sodium dodecyl sulfate (SDS)]. GST-ICER protein (500 ng) was added at the last washing step. After that, the washed beads were subjected to in vitro ubiquitination assay to determine CHIP Ub E3 ligase activity using the Ubiquitin-Protein Conjugation kit (Boston Biochem, Cambridge, MA, USA), following the manufacturer’s protocol. Briefly, the combined immunoprecipitated CHIP and GST-ICER were incubated with a ubiquitin and E1/E2 enzyme mixture in an energy buffer for 90 min at 37°C, with gentle shaking. The reaction was then stopped by adding 2X SDS loading buffer followed by Western blot analysis with an anti-ubiquitin antibody. Since bands over 300kDa were close to the stacking gel and were often not clearly resolved, we excluded bands over 300kDa from quantification. As shown in Fig.2, we found the dimer of GST-ICER around 70 kDa, but non-specific IgG bands dominated the area around 40-60kDa. Therefore, we selected the area from 70-280 kDa for quantification.

**Permanent coronary ligation surgery:** Myocardial ischemia was induced in the mouse by permanent ligation the left coronary artery: The mouse was placed onto a heating pad (half inch plexiglass between the animal and the heating pad). Oral intubation was established using a PE 90 tube (o.d. 1.27 mm) advanced slowly into the trachea. Mechanical P.I. ventilation (tidal volume of approximately 250 µl at 130 breaths/min) was begun. Maintenance anesthesia was 1.5% isoflurane. After intubation, a left thoracotomy was performed in the fourth intercostal space. The mouse heart was exposed, and the left coronary artery was ligated intramurally 2 mm from its ostial origin with 9-0 silk suture. The suture was secured to occlude the coronary artery. After confirming that the LV color change and ECG S-T segment elevation, the chest was closed with 6-0 coated vicryl suture, the skin was closed using 6-0 nylon, the anesthesia was stopped and the mouse was allowed to recover for several minutes before the endotracheal tube was removed. A sham operation involved an identical procedure, except that the suture was passed through the myocardium without occlusion. Animals were monitored by ECG during surgery. Aseptic
technique was used throughout. Animals were placed on a warming pad for all procedures to reduce the risk of hypothermia.

**Streptozotocin (STZ) injections:** Hyperglycemic mice (8-12 weeks) were made by a single dose intraperitoneal injection (IP) of freshly prepared STZ solution (150 – 200 mg/kg body weight in citrate saline, pH 4.5) using 26.5 Gauge needle. Diabetic status was confirmed by measuring blood glucose at day 7 of STZ injection.

**Analysis of apoptosis:** Cardiomyocyte apoptosis was measured by the terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) detecting in situ DNA fragmentation. TUNEL staining was performed using the In Situ Cell Death Detection Kit, Fluorescein (Cat # 11 684 795 910; Roche Diagnostics, IN, USA) as described previously16. For the counter staining of cardiomyocytes, cells were also stained with anti-cardiomyocyte-specific sarcomeric anti-actinin antibody. Briefly, cardiomyocytes or the coronal sections of frozen hearts were washed three times with cold PBS and fixed with a freshly prepared paraformaldehyde (4%) in PBS for 30 min, followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. Cardiomyocytes or heart sections were then incubated with blocking buffer (5 % goat serum in PBS) for 60 min to block nonspecific binding. Incubation with the primary antibody (anti-actinin antibody, 1:5000 dilution in 1% goat serum/PBS) was performed at 4°C overnight. After that, Cardiomyocytes or heart sections were washed three times with PBS. Then, secondary antibody labeled with Alexa Fluor 546 dye against mouse IgG1 (Molecular Probes; 1:2000 dilution) was added and incubated for 60 min at room temperature. After washing, cells were applied to TUNEL staining following the manufacturer’s instruction. Cells were mounted using mounting medium for fluorescence with DAPI (Vectashield, H-1200, Vector Laboratories, Inc. Burlingame, CA 94010). All of the images were taken on an Olympus BX51 upright fluorescence microscope equipped with an 40x objective lens. Quantification of the overlay image was done using ImageJ software (National Institute of Health). An average of 500 anti--actinin antibody positive cells from random fields were analyzed. All measurements were performed blinded and at least three independent experiments were performed.

**Echocardiographic analysis:** Echocardiographic analysis using M-mode was performed using a Vevo 2100 echocardiography machine from VisualSonics (Toronto Canada) equipped with a MS-550D probe. Echocardiography (M-mode image) of mouse left ventricle was obtained in un-anesthetized mice. LV function was measured in the parasternal short axis view at midlevel. %FS was assessed by measurement of the end-diastolic and end-systolic diameter (end diastolic diameter (Dd) – end systolic diameter (Ds))/end-diastolic diameter x100%). %EF was assessed by Dd3-Ds3/Dd3. We collected and averaged the data from 5 heart beats per trace, and three traces from each animal. The pooled data were analyzed for statistical significance.

**Assesment of infarct size:** The harvested hearts were fixed with 10% formalin for one day, dehydrated in ascending ethanol series, and embedded in paraffin. The tissues were sliced at 5 µm thickness from apex to basement into three transverse slices. The Sections were stained by Masson’s trichrome staining for a determination of infracted region. Slides were analyzed and photographed using an Olympus light microscope (Model BX41). The length of entire circumference and infarcted area were quantified using ImageJ software. The MI size was evaluated from each of three slides16.

**LC-MS/MS analysis of ERK5 phosphorylation by p90RSK:** Kinase reactions were performed in 1.5 mL LoBind tubes (Eppendorf, Hauppauge, NY). GST-ERK5 fusion protein (1 µg) was attached to glutathione-agarose resin. The beads (30 µL packed volume) were washed with 50 mM ammonium bicarbonate to remove any protease inhibitors left after protein purification. The beads were then resuspended in 100 µL of the kinase assay solution, and the kinase reaction was initiated by the addition
of 1 µg active RSK1 and 400 µM/L ATP. After 30 min of kinase reaction at 30°C, the reaction buffer was removed from the beads, which were then washed with 50 mM ammonium bicarbonate three times. For LC-MS/MS analysis, the GST-ERK5 fusion protein on beads was resuspended in 20 µL of solution containing 50 mM ammonium bicarbonate and 188 ng trypsin and was digested overnight at 37°C. The sample was reduced by 2 mM DTT for 30 min, with shaking at 60°C, alkylated by 10 mM iodoacetamide (IAA) for 30 min in the dark at room temperature (RT), and finally quenched by 10 mM cysteine for 30 min at RT in the dark. A second aliquot of 188 ng of trypsin was then added and digested for further 3 hr. Chymotrypsin digestion was performed in the identical manner. Digested samples were lyophilized and resuspended in 10 µL of 5% acetonitrile and 0.05% formic acid, and 15% of this peptide digest (1.5 µL) was loaded on a Magic C18 AQ (Michrom) nanospray tip, packed to 5 cm. This tip was loaded, using a pressure bomb and washed after installation on the HPLC with 5% methanol and 0.1% formic acid for 10 min with a flow rate of 600 nL/min (about 10 column volumes = 6.6 µL). The peptides were eluted and analyzed by an LC-MS/MS run, using a 5-15% methanol gradient over 2.5 min, followed by a 15-60% methanol gradient for 67 min, a 60% methanol isocratic step of 4 min, and ending with a 3 min 95% methanol step, with all solvents containing 0.1% formic acid. A full MS survey scan was performed every 3 seconds and the top 7 peaks were selected to produce an MS/MS fragmentation spectrum. The top three fragmentation spectra that experienced a neutral loss of 98 daltons were further fragmented to produce an MS/MS/MS spectrum.

The MS and fragmentation spectrum data was used in a Mascot search of the human proteome to identify peptide sequences modified by phosphate groups. The following search criteria were used for selecting fragmentation spectra that mapped to phosphorylated peptides: peptide tolerance = -0.8 to +0.5, a minimum ion score of 15, and a fragmentation spectrum containing fragment ions that either included or flanked the phosphorylated amino acid position. Representative fragmentation spectra are included in Figs.4B and 4C. Phosphorylation sites that mapped to the same ERK5 protein position in both the trypsin and chymotrypsin-treated samples were regarded as confirmed phosphorylation sites.

**Statistical Analysis:** Data are reported as mean ± S.D. Statistical analysis was performed with the GraphPad Prism program, version 4.00 (GraphPad Software, Inc. CA). Differences were analyzed with 1-way or 2-way analysis of variance (ANOVA) with repeated measures, followed by Tukey’s multiple comparison test. P values less than 0.05 are accepted as being statistically significant and indicated by one asterisk (*). Those less than 0.01 are indicated by two asterisks (**).
References
