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TNFα Shedding in Mechanically Stressed Cardiomyocytes is Mediated by Src Activation of TACE

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ABSTRACT

Synthesized by the heart under hemodynamic overloading (mechanical stress), TNFα exerts complex effects on the heart —beneficial as a membrane protein and detrimental as a secreted protein, which presents a dilemma in the treatment of congestive heart failure. We postulate that by selectively blocking mechanical stress-induced cardiomyocyte secretion of TNFα, a function of TNFα converting enzyme (TACE), the detrimental effect of TNFα can be mitigated. However, the mechanism through which mechanical stress activates TACE in cardiomyocytes is unknown. Here, we report a molecular mechanism that mediates TACE activation in mechanically stressed cardiomyocytes. We found that the non-receptor tyrosine kinase Src mediates TACE activation in mechanically stretched rat cardiomyocytes by phosphorylating the Tyr-702 residue within the intracellular tail of TACE. The rapid activation of Src in mechanically stretched cardiomyocytes is followed by TACE phosphorylation on Tyr-702, leading to activation of p38 MAPK, a kinase that is an effector of TNFα receptor activation. Pharmacological inhibition or silencing of Src attenuated stretch-induced TACE phosphorylation on Tyr-702 and p38 activation. Overexpression of a TACE mutant in which Tyr-702 was replaced by alanine (TACE-Y702A) attenuated stretch-induced TNFα release from cardiomyocytes as well as activation of p38. These data suggests that Src mediates TACE activation in mechanically stressed cardiomyocytes and this mechanism could be exploited for specific blockade of TNFα secretion and its detrimental effects in congestive heart failure. J. Cell. Biochem. 116: 559–565, 2015.

KEY WORDS: CARDIOMYOCYTES; Src; TACE; TNFα; p38 MAPK; MECHANICAL STRETCH

The heart adapts to a sustained increase in hemodynamic overload through a remodeling process that results in cardiac hypertrophy, and eventually leads to left ventricle (LV) dilation and heart failure (Lorell and Carabello, 2000). It is well established that the heart expresses TNFα as part of its response to overloading stress, and this sustained increase in TNFα expression due to overloading directly contributes to left ventricular remodeling resulting in cardiomyocyte hypertrophy, LV dilation and congestive heart failure (CHF) [Mann, 2002]. As a short-term treatment in CHF patients, the neutralization of TNFα with a soluble TNFα receptor (etanercept) improved symptoms for up to 3 months [Bozkurt et al., 2001]. However, longer-term treatment with anti-TNFα reagents exacerbated CHF in a dose- and time-dependent manner [Mann, 2005]. TNFα has long been recognized as a pleiotropic cytokine with diverse and sometimes conflicting effects (Tracey and Cerami, 1992). The paradoxical failure of clinical trials employing anti-TNFα reagents suggests that TNFα also has beneficial effects in the failing heart, which gives rise to a dilemma in the therapy of CHF. Resolving this dilemma could significantly improve the treatment of CHF.

After being synthesized by the cell, TNFα is transported to plasma membrane as a pro-protein (26 kDa). The pro-TNFα is cleaved and TNFα is shed (secreted) as a 17 kDa protein by TACE, a member of the ADAM family of disintegrin metalloproteinases (also known as ADAM17) [Blobel, 1997; Moss et al., 1997]. In addition, TACE is responsible for shedding of a number of other proteins including L-selectin, TGFα, type 1 TNFα receptor, and EGF [Peschon et al., 1998; Black, 2002]. Importantly, secreted TNFα mediates a dilated LV phenotype, but membrane TNFα stimulates a concentric LV

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hypertrophy phenotype [Diwan et al., 2004], suggesting that secreted TNFα is responsible for its detrimental effects in the failing heart, while membrane TNFα mediates the beneficial effects. Therefore, we reason that TNFα shedding rather than TNFα per se should be targeted for the therapy of CHF. In deed, TACE is implicated in the pathology of CHF. In pathophysiological contexts where LV dilation and dysfunction occur, TACE expression is upregulated in the heart along with TNFα expression [Satoh et al., 1999]. Experimentally induced overloading stimulates TACE activity in mouse heart, and this effect is amplified in absence of the physiological TACE inhibitor TIMP3 [Kassiri et al., 2005]. In addition, mice with elevated TACE activity develop spontaneous dilated cardiomyopathy [Fedak et al., 2004]. In fact, pharmacological inhibition of TACE prevents the transition of the LV hypertrophy to LV dilation in mice that overexpress TNFα in myocardium [Dibbs et al., 2003]. However, existing pharmacological inhibitors of TACE that target the extracellular active center of the enzyme are too toxic for human use [Murumkar et al., 2013]. We hypothesize that mechanical stress (overloading) activates TACE in cardiomyocytes through a post-translational mechanism, and by blocking the mechanotransduction signaling that activates TACE it is possible to selectively block TNFα secretion induced by mechanical stress and thereby mitigate the detrimental effects of TNFα. However, the mechanism through which TACE is activated by mechanical stress in cardiomyocytes is unknown.

TACE possesses the structural features of substrates of the non-receptor tyrosine kinase Src. The intracellular tail of TACE contains a potential SH3-binding motif (731-PAPQTPGR-738) that is adjacent to a highly conserved putative tyrosine phosphorylation motif (696-KKLDKQ*Y*ESL-705) [Moss et al., 1997]. The Tyr-702 residue within the putative tyrosine phosphorylation motif has been recently identified in skeletal myoblasts as a bona fide phosphorylation site targeted by Src, and phosphorylation of Tyr-702 activates TACE release of TNFα [Niu et al., 2013]. However, whether a similar mechanism exists in cardiomyocytes to mediate TACE activation by mechanical stress remains unknown. Src expression and activity are increased in failing heart [Vitello et al., 2012]. Particularly, Src is activated by mechanical stress in cardiomyocytes in vitro and in vivo [Franchini et al., 2000; Takeishi et al., 2001; Aikawa et al., 2002]. In addition, Src activation in mechanically stretched cardiomyocytes mediates the activation of p38 MAPK [Aikawa et al., 2002] that is responsible for a number of cardiac pathologies [Rose et al., 2010], although the underlying mechanism of Src activation of p38 is unknown. Given that TNFα is a potent activator of p38 in cardiomyocytes [Craig et al., 2000; Dhingra et al., 2007], we hypothesize that in mechanically stressed cardiomyocytes Src activates TACE through the phosphorylation of TACE on Tyr-702, resulting in increased TNFα shedding and p38 activation, which mediates the detrimental effect of TNFα. In the current study we test this hypothesis in mechanically stretched cardiomyocytes and report data that support this hypothesis.

MATERIALS AND METHODS

CULTURE AND STRETCH OF NEONATAL RAT CARDIOMYOCYTES

Experimental protocol involving the use of neonatal rats was approved in advance by the Institutional Animal Welfare Committee (AWC) at University of Texas Health Science Center at Houston. Cardiomyocytes from 2-day-old Sprague-Dawley rats (Charles River Laboratories) were purified by Percoll density gradient centrifugation, 4×10^5 cells were plated in six-well Bioflex® plates (Flexcell International, Hillsborough, NC) that were coated with collagen I, and incubated overnight in DMEM/Ham's nutrient mixture F-12 (1:1) supplemented with 10% FBS. The medium was then replaced with fresh medium supplemented with 5% horse serum, and then cultured for an additional 24 h. At this point, cells were placed in serum free DMEM medium and cultured overnight. When indicated, adenovirus was included in the medium. Next morning, cells were placed in fresh serum free DMEM medium for two hours before constant 10% or 20% global stretch was initiated using Flexcell® FX-5000™ Tension System (Flexcell International, Hillsborough, NC). Parallel sets of non-stretched cardiomyocytes were used as controls. Stretch was maintained for a designated period of time under normal cell culture conditions (37 °C with 5% CO_2 in a humidified incubator). When indicated, the Src family tyrosine kinase (SFK) inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-days]pyrimidine (PP2) or its negative control PP3 (Sigma-Aldrich), dissolved in DMSO (0.1% final concentration), was added to the medium (final concentration 10 μM) 30 min prior to and were maintained during stretch.

WESTERN BLOT ANALYSIS

Cardiomyocytes were lysed by votexing at 4 °C for 1 min in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate that was supplemented with 1 μM BB-94, 1:100 protease inhibitor cocktail (Sigma-Aldrich) containing AEBSF, pepstatin A, E-64, bestatin, leupeptin and aprotinin, and 1:100 phosphatase inhibitor cocktail (Sigma-Aldrich) containing sodium vanadate, sodium molybdate, sodium tartrate, and imidazole plus 1 mM NaF. Insoluble cellular debris was removed by centrifugation at 16,000 g (4 °C) for 10 min. The supernatant was collected, and protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with BSA as a standard. The samples were heated at 95 °C for 5 min in Laemmli sample buffer (Bio-Rad) containing 10 mM dithiothreitol. SDS-PAGE and nitrocellulose membrane transfer were performed using Bio-Rad mini format electrophoresis system. Antibody-detected proteins on blotted membrane were visualized by enhanced chemilumescent (ECL) and quantified by densitometry using Kodak 1D Image Analysis software (Eastman Kodak, Rochester, NY) in optical density (semi-quantitative) and normalized to loading control (total protein or GAPDH where appropriate). Antibodies for Src, pY416-Src, p38, and phosphorylated p38 (T181/Y182) were from Cell Signaling and antibody for TACE was from QED Bioscience. Rabbit polyclonal antibody against pY702-TACE was raised as previously described [Niu et al., 2013].

TRANSFECTION OF siRNA

The on-target smart pool siRNA specific for Src and control siRNA were purchased from Dharmacon (Denver, CO) and Ambion (Austin, TX), respectively, and were introduced into cardiomyocytes by using the Lipofectamine RNAiMAX reagent (Invitrogen) according to manufacturer's protocol.

RECOMBINANT ADENOVIRUS

Recombinant adenovirus encoding murine wild type TACE and the TACE mutant TACE-Y702A were generated as previously described [Niu et al., 2013]. Cardiomyocytes were transduced with the adenovirus at approximately 100 MOI, with adenovirus encoding green fluorescence protein (GFP) cDNA (prepared by The Vector Development Core of Baylor College of Medicine) as control.

DETERMINATION OF TNFα CONCENTRATION IN CULTURE MEDIUM

Culture media (serum-free DMEM) from cardiomyocytes with or without stretching for 2 h were collected and concentrated with a spin concentrator from Millipore (10 K pore size). TNFα concentration was then determined by using an ELISA kit (R&D Systems) according to the manufacturer's protocol.

STATISTICAL ANALYSIS

All experiments were repeated at least three times as indicated. Data are presented as means ± SE and were analyzed with Student's t test or 1-way ANOVA, as appropriate, using SigmaStat software (Systat Software, Inc., San Jose, CA). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

To evaluate cardiomyocyte response to mechanical stress neonatal rat cardiomyocytes grown on silicon membrane in serum free medium were subjected to 10% single strain global stretch, which is considered a moderate level of stress and mimics the overloading stress that cardiomyocytes are subjected to in CHF (Mann and Kent, 1989). The stretch resulted in a rapid activation of Src within 5 min as indicated by phosphorylation of its Tyr-416 residue that was detected by Western blot analysis. The activation reached the peak level around 15 min and then gradually subsided (Fig. 1A). Utilizing a previously described antibody raised against TACE phosphorylated on Tyr-702 [Niu et al., 2013], a similar increase in TACE phosphorylation on Tyr-702 was observed, which reached the peak level behind Src around 30 min (Fig. 1B). Mechanical stretch of cardiomyocytes is known to activate p38 MAPK in a Src-dependent manner through an unknown mechanism [Aikawa et al., 2002]. Therefore, we monitored p38 activity as a downstream effector of Src in stretched cardiomyocytes and observed its activation that peaked around 60 min following TACE phosphorylation on Tyr-702 (Fig. 1C). These observations revealed a rapid activation of TACE in mechanically stressed cardiomyocytes, and established a time line of the sequential activation of Src, TACE and p38.

Next, we attempted to determine whether there is a causal relationship connecting the above sequential events. To assess whether Src mediates TACE phosphorylation on Tyr-702 in response to mechanical stress, cardiomyocytes were treated with a pharmacological inhibitor of SFK, PP2, or its non-functional analog, PP3, and stretched for 30 min. PP2 specifically blocked stretch-stimulated TACE phosphorylation on Tyr-702 (Fig. 2A). To ascertain a critical role of Src in this process, cardiomyocytes were transfected with Src-specific siRNA to silence the gene expression. We observed that in transfected cardiomyocytes p38 activation by stretch was delayed to peaking around 120 min, possibly due to cell stress caused by the transfection procedure. Nevertheless, in Src-deficient cardiomyocytes stretch-induced phosphorylation of TACE on Tyr-702 (Fig. 2B) as well as p38 activation (Fig. 2C) were significantly diminished. These

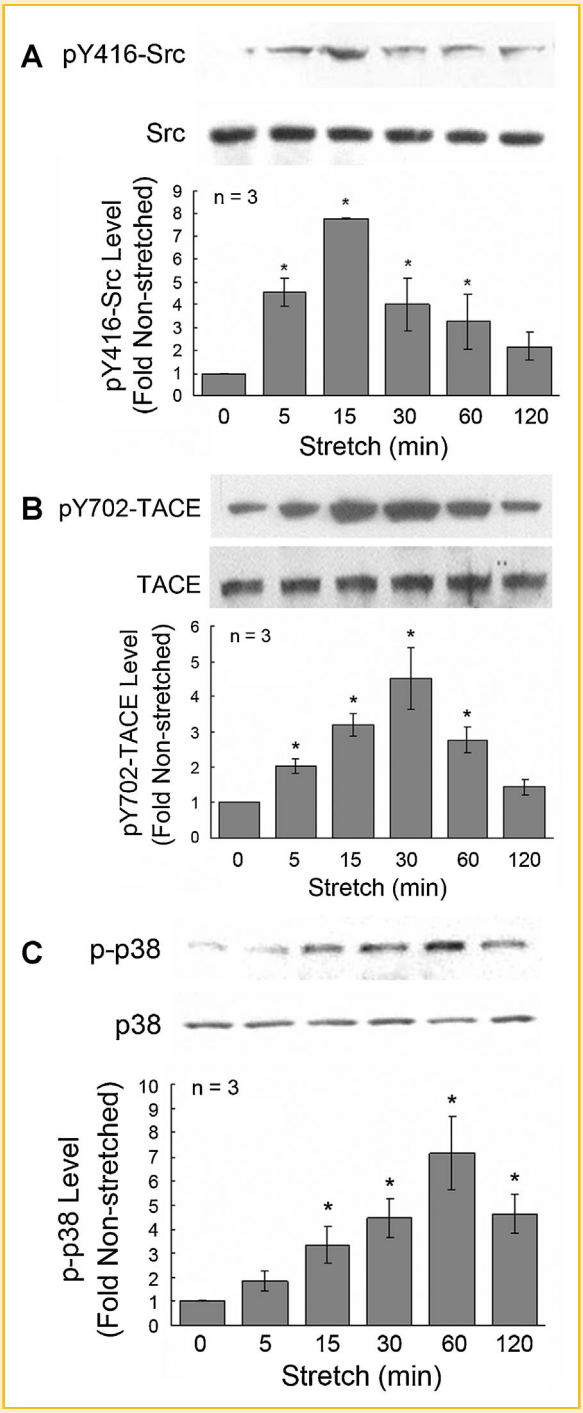

Fig. 1. Mechanical stretch of cardiomyocytes rapidly induces TACE phosphorylation on Tyr-702 and p38 activation following Src activation. Cardiomyocytes were stretched for indicated periods and lysed for Western blot analysis of Src phosphorylation on Y416 (A), TACE phosphorylation on Y702 (B) and p38 phosphorylation on T181/Y182 (C). Data was analyzed by ANOVA ($P < 0.001$), and * indicates a difference from non-stretched control.

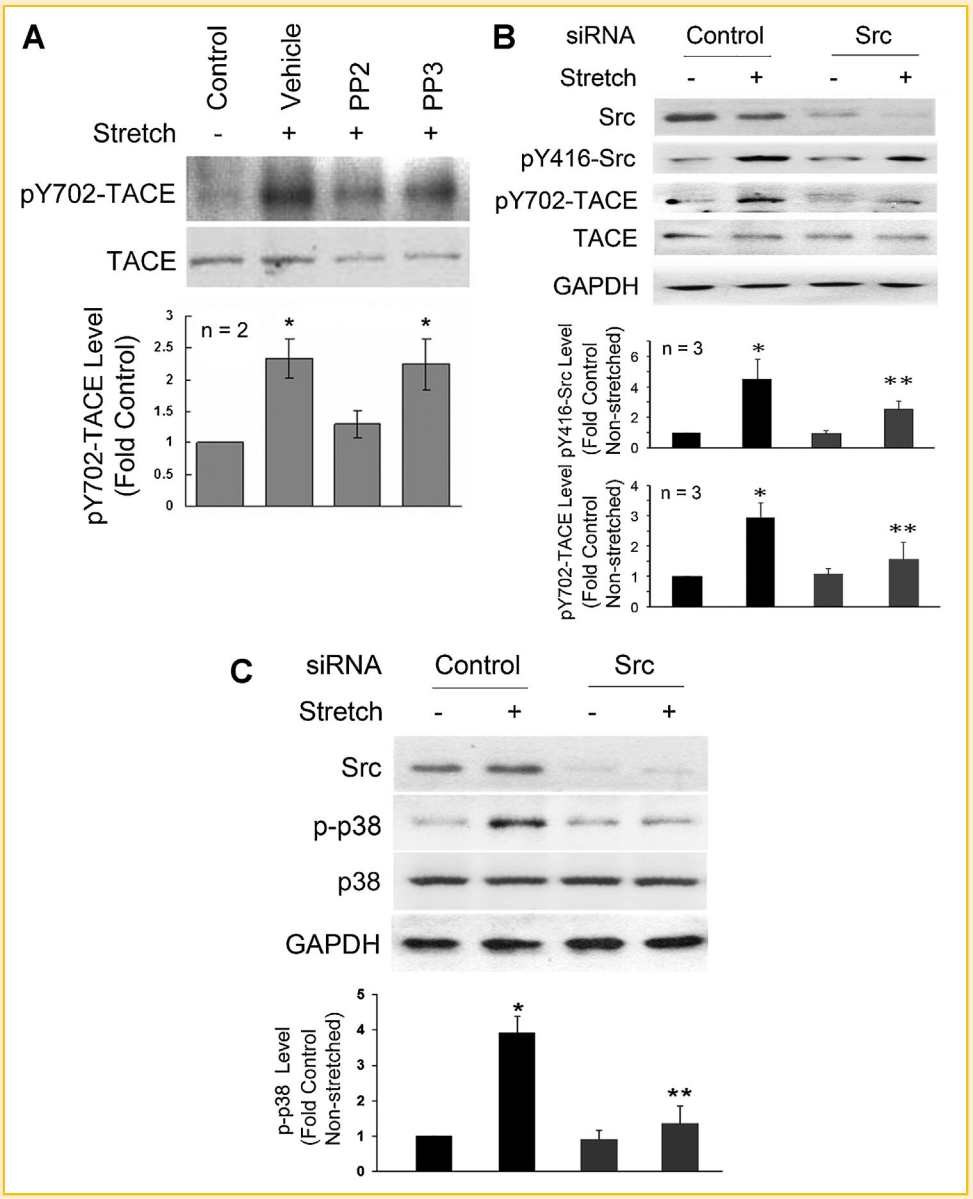

Fig. 2. Mechanical stretch-induced TACE phosphorylation on Tyr-702 and p38 activation are dependent on Src. (A) Cardiomyocytes were treated with 10 μM of PP2, PP3, or vehicle (0.1% DMSO) as indicated 30 min prior to a 30 min stretch. Phosphorylation of TACE on Tyr-702 and total TACE were determined by Western blotting. Data was analyzed by ANOVA, * indicates a difference from control ($P < 0.05$). (B) Cardiomyocytes were transfected with control or Src-specific siRNA, and stretched for 30 min with time-matched non-stretched control. Src expression, Src phosphorylation on Y416 and TACE phosphorylation on Tyr-702 were analyzed in cell lysate by Western blotting. (C) Another set of transfected cardiomyocytes was similarly stretched for 120 min and analyzed for p38 phosphorylation on T181/Y182. Data was analyzed by ANOVA, * indicates a difference from non-stretched cells transfected with control siRNA, and ** indicates a difference from stretched cells transfected with control siRNA ($P < 0.05$).

observations support a role for Src in mediating TACE phosphorylation on Tyr-702 in mechanically stressed cardiomyocytes, and verify that Src is required for mechanical activation of p38 in cardiomyocytes.

To investigate whether stretch-stimulated TACE phosphorylation on Tyr-702 is critical to TACE-mediated TNFα release and subsequent activation of p38 in cardiomyocytes, we transduced the cells with an adenovirus encoding a mutant TACE in which Tyr-702 was replaced by alanine (TACE-Y702A), and with an adenovirus encoding the wild type TACE or the green fluorescence protein (GFP) as controls. We observed that transduction weakened cardiomyocyte response to stretch, possibly due to the adverse effects of coxsackievirus and adenovirus receptor (CAR) activation [Kallewaard et al., 2009] which is likely to stress the cells, such that 20% stretch was necessary to induce similar responses induced by 10% stretch in non-transduced cells. Therefore, 20% stretch, which is a standard level of mechanical stress used in similar studies [Sadoshima et al., 1992; Aikawa et al.,

2002] that does not cause cell injury [Sadoshima et al., 1992] was applied to transduced cells. As shown in Figure 3, overexpressed TACE-Y702A mutant blocked stretch-induced TACE phosphorylation on Tyr-702 despite an intact Src activation (Fig. 3A), resulted in a reduction in TNFα release into the culture medium (Fig. 3B) as well as p38 activation in cardiomyocytes (Fig. 3C). Therefore, stretch-induced TACE phosphorylation on Tyr-702 mediates its release of TNFα and subsequent activation of p38 in cardiomyocytes. These data demonstrate that in cardiomyocytes mechanical stress activates TNFα shedding by Src-mediated activation of TACE through the phosphorylation of its Tyr-702 residue.

DISCUSSION

The current study demonstrates for the first time in cardiomyocytes that mechanical stress-induced TNFα shedding is mediated by Src activation of TACE via phosphorylation on its Tyr-702 residue. These findings reveal the mechanism of the rapid activation of TNFα shedding by mechanically stressed cardiomyocytes, and provide a potential therapeutic strategy for congestive heart failure.

Given the rapid nature of TACE activation in mechanically stressed cardiomyocytes, the current study focused on post-transcriptional regulation of TACE, which is highly complex. The

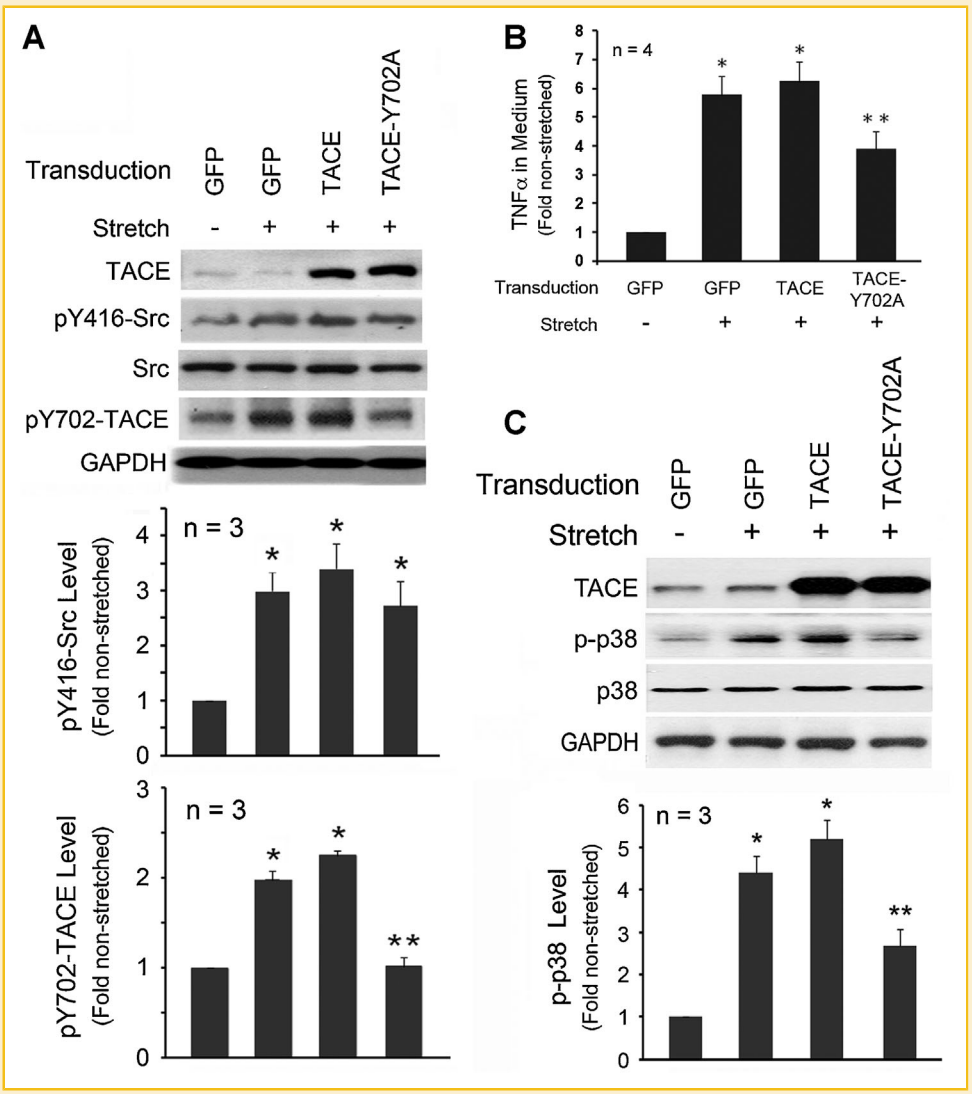

Fig. 3. Mechanical stretch-induced TACE phosphorylation on Tyr-702 is critical to TACE-mediated TNFα release and p38 activation. Cardiomyocytes were transduced with adenovirus encoding murine wild type TACE or the TACE-Y702A mutant, and with adenovirus encoding GFP as control. (A) Transduced cardiomyocytes were stretched for 30 min with time-matched non-stretched cells that was transduced with adenovirus encoding GFP as control. Harvested cells were lysed for Western blot analysis of expression of the recombinant TACE, phosphorylation of Src on Tyr-416 and TACE on Tyr-702. (B) Another set of transduced cardiomyocytes was similarly stretched for 120 min. The cell culture medium was concentrated after filtration and analyzed for TNFα content using ELASA. (C) Cell lysate of cardiomyocytes stretched for 120 min above was prepared and analyzed by Western blotting for p38 activation. Data were analyzed by ANOVA, * indicates a difference from non-stretched cells transduced with adenovirus encoding GFP, and ** indicates a difference from stretched cells transduced with adenovirus encoding GFP.

intracellular tail of TACE contains multiple phosphorylation sites including Thr-735, Ser-791 and Ser-819, which are regulated by growth factors through such kinase as ERK1/2 MAPK [Diaz-Rodriguez et al., 2002; Fan et al., 2003; Soond et al., 2005]. However, the functional consequences of phosphorylation at these sites are not well understood. As a protein tyrosine kinase, Src is well-established as a key molecule that mediates mechanotransduction in various types of load-sensitive cells [Torsoni et al., 2003; Wang et al., 2005; Rangaswami et al., 2010; Niu et al., 2013]. Our data demonstrate that Src-mediated phosphorylation of Tyr-702 activates TACE to shed TNFα in mechanically stressed cardiomyocytes, thereby, contributes to the cellular adaptation to overloading in the heart.

TNFα is a potent activator of p38 in cardiomyocytes [Craig et al., 2000; Dhingra et al., 2007]. Because both membrane and secreted TNFα are biologically active, the basal activity of p38 MAPK in non-stretched cardiomyocytes is likely influenced by both forms of TNFα. However, when TACE is activated in the 2 h stretch, increased shedding of TNFα would result in a reduction of membrane TNFα before newly synthesized TNFα replenishes the membrane TNFα pool, therefore, secreted TNFα is responsible for p38 activation during this window of time. Another noteworthy issue is that the lapse between the activation of TACE and p38 observed is likely due to the time required for TACE-released TNFα to accumulate to certain levels and the sensitivity of our detection method. Further, p38 can be activated by multiple stimuli and activation of p38 in stretched cardiomyocytes may result from the release of additional mediators. Activated p38 is thought to mediate a wide spectrum of cardiac pathologies, including hypertrophy, myocardial infarction, as well as systolic and diastolic heart failure [Rose et al., 2010]. Src is implicated in the pathology of heart diseases [Vitello et al., 2012]. Particularly, it was linked to p38 activation in mechanically stretched cardiomyocytes [Aikawa et al., 2002], however, the underlying mechanism was unknown. Our data revealed the mechanism through which Src mediates p38 activation in cardiomyocytes through the activation of TACE. However, from the therapeutic point of view, Src inhibition may not be a preferred strategy for the intervention of CHF, considering that Src mediates a wide range of signaling events in various types of cells. On the other hand, blocking TACE activation in response to mechanical stress could be a more selective therapeutic strategy.

In addition to activating p38 MAPK, secreted TNFα may alter the shedding of other cell surface molecules [Jurisic et al., 2011], which may also be attenuated by the blocking of TACE activation.

It is notable that while overexpressed TACE Y702A mutant effectively blocked TACE phosphorylation on Tyr-702, TNFα shedding and p38 activation were attenuated but not totally blocked (Fig. 3). It is possible that additional mechanisms may be involved in TNFα shedding in response to mechanical stress. For example, other members of the ADAM family, ADAM 10 [Mezyk-Kopec et al., 2009] and ADAM19 [Zheng et al., 2004], are also capable of shedding TNFα although the physiological and pathological significance of these ADAMs in TNFα shedding is unclear. In addition, phosphorylation of Y702 may not be the only mechanism that regulates TACE activity in response to stretch in cardiomyocytes, because TACE can be activated without the presence of the cytoplasmic tail, presumably by protein kinase C [Reddy et al., 2000]. On the other hand, Src silencing effectively blocked p38 activation as well as TACE phosphorylation on Tyr-702 (Fig. 2), suggesting the possibility that Src mediates TNFα shedding by activating multiple sheddases or TACE mediates the release of other p38-activating substrate(s). Nevertheless, our data demonstrates in principle that it is possible to selectively block the signaling pathway that mediates mechanical stress-induced TNFα shedding by cardiomyocytes.

Despite demonstrated effectiveness of TACE inhibition in preventing TNFα-induced LV dilation in mice [Dibbs et al., 2003], existing pharmacological inhibitors of TACE are too toxic for human use, partially due to their indiscriminative inhibition of TACE activation by blocking its extracellular active center [Murumkar et al., 2013]. In addition, inhibition of TACE may affect other molecules and therefore have side effects. The discovery of the mechanism of TACE activation by mechanical stress affords us an opportunity to selectively attenuate TNFα shedding in response to mechanical stress without affecting the basal activity of TACE or its activation by other types of stimuli systemically. The Src-targeted tyrosine phosphorylation motif (696-KKLDKQ*Y*ESL-705) in TACE is highly unique – it is found only in TACE by a BLAST search. Thus, it could be a relatively specific drug target.

Although the current study shows that mechanical stress activates TNFα shedding by cardiomyocytes through a similar mechanism as found in skeletal myoblasts [Niu et al., 2013], the functionality of TNFα shedding is quite different in the two types of cells. In skeletal muscle, TNFα shed by myoblasts promotes myogenesis and muscle regeneration [Chen et al., 2007; Zhan et al., 2007]. In contrast, TNFα shed by cardiomyocytes is considered a pathological mediator for heart failure [Diwan et al., 2004]. Thus, the novelty of our findings reported here lies in the pathological implication for CHF.

Taken together, our data suggests that the adaptation of cardiomyocytes to mechanical stress involves the activation of a Src-TACE-TNFα-p38 MAPK signaling pathway, and the activation of TACE by Src is a potential therapeutic target of CHF.

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