ASK1 Associates with Troponin T and Induces Troponin T Phosphorylation and Contractile Dysfunction in Cardiomyocytes

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There is increasing support for the idea that excessive production of proinflammatory mediators such as tumor necrosis factor (TNF) and reactive oxygen species (ROS) contribute to the pathogenesis of cardiac dysfunction. However, the mechanisms by which cytokine/ROS production mediates cardiac dysfunction have not been established. Given that apoptosis signal-regulating kinase 1 (ASK1) is highly expressed in cardiac muscle and that ASK1 is an important mediator in the signaling pathways induced by tumor necrosis factor, interleukin-1, and ROS, we used the yeast two-hybrid system with ASK1 as bait to identify ASK1 substrates from a human heart cDNA library. The cDNA encoding the cardiac troponin T (cTnT) was isolated. ASK1 specifically interacted with cTnT, but not cTnI, *in vitro* and *in vivo* via the C-terminal ASK1 domain. ASK1 specifically phosphorylated cTnT *in vitro* and *in vivo*. Mutations in cTnT (T194/S198) at an ASK1-phosphorylation consensus sequence significantly reduced phosphorylation by ASK1. ROS-induced ASK1 activation, cTnT phosphorylation, and contractile dysfunction in cardiomyocytes showed similar kinetics. Moreover, overexpression of constitutively active ASK1 induces cTnT phosphorylation and inhibits shortening and calcium transient in adult cardiomyocytes. We conclude that ASK1 plays an important role in regulation of cardiac contractile function by phosphorylating cTnT and may participate in cytokine/ROS-induced pathogenesis of cardiomyopathy and heart failure. (*Am J Pathol* 2003, 163:243–251)

There is increasing support for the idea that excessive production of proinflammatory mediators such as tumor necrosis factor (TNF)- α , interleukin-1 β , and reactive oxygen species (ROS) contributes to myocardial infarction, cardiomyopathy, arterial hypertension, and heart failure.^{1–6} Many aspects of the syndrome of heart failure

phenotype can be explained by the known biological effects of proinflammatory cytokines.⁷ Several experimental studies have shown that ROS reduce contractility of isolated myocytes and impair cardiac function.^{6,8–11} It is well established that ROS activate kinase cascades leading to phosphorylation of many substrates.^{12,13} However, the mechanisms by which proinflammatory cytokines/ROS mediate cardiac dysfunction have not been established.

An important pathophysiological mechanism for cardiac dysfunction is decreased myofibrillar ATPase (MgATPase).^{14,15} Cardiac troponin (cTn) and tropomyosin regulate the myofibrillar ATPase by modulating calcium sensitivity.¹⁶ Tn consists of three subunits, each of which performs specific functions. TnC binds to Ca²⁺, TnI inhibits the ATPase activity of actomyosin, and TnT provides a site for the binding of Tn to tropomyosin. The functional significance of Tn-tropomyosin regulatory complex is suggested by genetic mutations in familial hypertrophic cardiomyopathy.¹⁷ It has also been shown that phosphorylations of cTnI and cTnT are critical regulatory events that modulate Ca²⁺-stimulated myofibrillar MgATPase activity in the normal heart.^{18–20} Phosphorylation of cTnI by protein kinase A has been associated with reduced sensitivity of the myofibrillar MgATPase to Ca²⁺.²¹ Earlier investigations suggested that phosphorylation of cTnI and cTnT by protein kinase C (PKC) decreases actin-myosin interaction and reduces maximal myofibrillar MgATPase activity.^{18–20} Thus, phosphorylation-dependent changes in the regulatory effects of the troponin-tropomyosin complex on actin and myosin may explain the depressed myofibrillar MgATPase activities in cardiac dysfunction. However, the kinase(s) stimulated by ROS and proinflammatory cytokines that increase cTn phosphorylation have not been determined.

ASK1 is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) superfamily that has been implicated in the intracellular signaling cascades triggered by stress stimuli.^{22–26} ASK1 consists of the N-terminal regulatory domain, the internal kinase domain,

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and the C-terminal regulatory domain. The C-terminal domain has been shown to bind several regulatory proteins such as the activator TRAF2²⁷ and the inhibitor 14-3-3.^{25,26,28} Recently, data from ASK1-deficient mice demonstrate a specific role for ASK1 in TNF- and ROS-induced intracellular signaling.²³ TNF- α and ROS activate ASK1, in part, by inducing homodimerization of ASK1 and by dissociating regulatory proteins.^{24,26,28,29} Because ASK1 appears to be an essential mediator for ROS and TNF signals and it is highly expressed in the heart,³⁰ we investigated its role in cardiac function. We demonstrate a novel function of ASK1 in regulating cTnT phosphorylation, myosin MgATPase activity, and cardiac contractile function.

Experimental Procedures

Plasmid Construction

ASK1- Δ N (a constitutively active form of ASK1 with deletion of the inhibitory N-terminal domain) was amplified by polymerase chain reaction using a 5' primer with *Nde*I site and a 3' primer with *Sal*I. The polymerase chain reaction product was inserted into *Nde*I and *Sal*I sites of the expression vector pAS2.1 (Clontech, Palo Alto, CA) to generate pAS-ASK1- Δ N in which ASK1- Δ N was fused in-frame with the DNA-binding domain of yeast transcriptional activator GAL4. The full-length human cTnT cDNA (accession no. NM000364, TNNT2, cardiac troponin T2) was amplified by polymerase chain reaction from a human heart cDNA library (Clontech) using a 5' primer with *Eco*RI site and a 3' primer with *Xho*I site. The polymerase chain reaction was cloned into mammalian expression Flag-vector (Flag-cTnT) or bacterial expression pGEX-kG vector (GST-cTnT). Similar expression constructs for human cTnI (accession no. NM000363) were also constructed. The mutant cTnT (T194A and S198A) was constructed by site-directed mutagenesis using Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the protocol of the manufacturer. A pair of primers was used to introduce these desired mutations. The sense primer was: 5'-GAA GCA GGC CCA GGC AGA GCG GAA AGC TGG GAA GAG GCA G-3' (the G is mutated from A and the GC is mutated from AG).

Yeast Two-Hybrid Screening

ASK1- Δ N bait was used to screen a pretransformed human heart cDNA library (Clontech). The yeast two-hybrid screening was performed according to the instructions of the manufacturer (Clontech). In brief, the yeast strain AH109 harboring pAS-ASK1- Δ N was mated with Y190 harboring a human heart cDNA library. Mating zygotes were selected on synthetic dropout agar plates lacking Trp, Leu, His, and Ade (QDO). Yeast colonies were transferred onto a nylon membrane and processed by the β -galactosidase filter assay. Plasmids from positive colonies were isolated and retransformed into the yeast strain Y190 with either pAS2.1 or pAS-ASK1- Δ N to confirm that growth on QDO and β -gal was ASK1- Δ N-depen-

dent. The cDNA inserts from true positive clones were subjected to DNA sequencing with a dye terminator cycle sequencing kit (University of Rochester core facility).

Preparation of Contractile Adult Rat Cardiomyocytes

The method for cardiomyocytes isolation from adult rats has been described previously.³¹ Briefly, isolated cardiomyocytes were prepared using a Langendorff setup with retrograde perfusion of collagenase solution (type II; Worthington, Lakewood, NJ). This enzyme solution was recirculated through the heart for ~30 minutes. The isolated cardiomyocytes were kept in standard solution that contained (in mmol/L): 140 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid (HEPES), 11 glucose, pH 7.4, at 37°C with NaOH. Isolated cells were used for experiments on the same day.

GST Pull-Down Assay

GST-cTnT protein expression, purification, and GST pull-down assay were performed as described previously.^{28,29}

cTnT Phosphorylation in Vitro and in Vivo

cTnT phosphorylation *in vitro* by ASK1 was performed as follows: ASK1- Δ N expression plasmid was transfected into 293T cells and ASK1- Δ N protein was immunoprecipitated with anti-Flag. Ten μ g of native cTn protein complex (cTnT/I/C) purified from human heart tissue (Research Diagnosis) or GST-cTnT (purified in the lab) was mixed with the ASK1- Δ N immunoprecipitate in the kinase buffer (20 mmol/L HEPES, pH 7.6, 20 mmol/L MgCl₂, 25 mmol/L β -glycerophosphate, 100 μ mol/L sodium orthovanadate, 2 mmol/L dithiothreitol, 20 μ mol/L ATP) containing 1 μ l (10 μ Ci) of [γ -³²P] ATP. The sample was incubated at 30°C for 30 minutes followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

cTnT phosphorylation *in vivo* by H₂O₂ in cardiomyocytes or by overexpressed ASK1- Δ N in 293T cells was examined. For cardiomyocytes, cells were treated with 100 μ mol/L of H₂O₂ for 0 to 15 minutes as indicated and phosphorylation of endogenous cTnT was determined. For 293T, cells were transfected with Flag-cTnT and Flag-ASK1- Δ N and phosphorylation of Flag-cTnT was determined at 24 hours after transfection. cTnT phosphorylation was performed as following: culture medium was removed and cells were washed three times with phosphate-free Krebs-Henseleit buffer solution (118 mmol/L NaCl, 4.0 mmol/L KCl, 1.2 mmol/L MgCl₂, 1.1 mmol/L K₂HPO₄, 25 mmol/L NaHCO₃, 5.0 mmol/L glucose, 20 mmol/L HEPES, pH7.4) supplemented with 1.8 mmol/L of CaCl₂ and 0.1% bovine serum albumin. Each culture plate was then added with 0.3 ml of the same medium containing carrier-free [³²P] orthophosphate (0.3 mCi/ml) and incubated at 37°C for 2.5 hours. Medium was re-

moved and the cells were washed with ice-cold wash buffer (150 mmol/L NaCl, 20 mmol/L HEPES, pH 7.4), solubilized in 100 μ l of 0.1% SDS, 6.6% glycerol, 3.3% 2-mercaptoethanol, 65 mmol/L Tris-HCl, pH 6.8, and frozen at -20°C . cTnT phosphorylation was analyzed by immunoprecipitation with anti-Flag or anti-cTnT followed by SDS-PAGE and autoradiography.

ASK1 Kinase Assays

Cardiomyocytes were treated with H_2O_2 (100 $\mu\text{mol/L}$) for 0 to 15 minutes. Cells were lysed in cold lysis buffer and ASK1 activity was measured by an *in vitro* kinase assay using GST-MKK4 as a substrate as described.²⁹

Lentiviral Vector Construction, Production, and Assay

A dual cDNA expression cassette vector was constructed to co-express Flag-tagged ASK1- ΔN with GFP, on a bi-*cis*-tronic mRNA, linked by IRES in the pHR⁺-CMV (a gift from Dr. I. Verma, The Salk Institute for Biological Studies, La Jolla, CA). The viral vector was prepared as previously described.^{32,33} The final pellet was resuspended in 0.05% of the starting volume in sterile phosphate-buffered saline (PBS) containing 4 $\mu\text{g/ml}$ of polybrene. Stocks were titered as described above and stored frozen at -80°C .

Transfection and Viral Infection

Transfection of 293T was performed by Lipofectamine according to the manufacturer's protocol (Life Technologies, Inc., Grand Island, NY). Lentiviral infection of cardiomyocytes was performed at multiplicity of infection (MOI) of 50 for 24 hours as described.³³

Immunoprecipitation and Immunoblotting

Cells were washed twice with cold PBS and lysed in 1.5 ml of cold lysis buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Triton X-100, 0.75% Brij 96, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 mmol/L sodium pyrophosphate, 10 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin, 2 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L ethylenediaminetetraacetic acid) for 20 minutes on ice. Immunoprecipitation and immunoblotting were performed as described.²⁹ with anti-ASK1 (H300; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cardiac troponin T (Santa Cruz Biotechnology), anti-Flag M2 (Sigma Chemical Co., St. Louis, MO) and anti-HA (Roche Diagnostics, Indianapolis, IN) as indicated.

Indirect Immunofluorescence Confocal Microscopy

Fixation, permeabilization, and staining of cultured rat adult cardiomyocytes were performed as described pre-

viously with a modification.³⁴ Briefly, cultured rat adult cardiomyocytes were rinsed in PBS and fixed for 15 minutes in 3% formaldehyde. The cells were then permeabilized for 15 minutes with 0.2% Triton X-100 followed by drying on a glass slide. The primary antibodies (goat anti-cTnT and rabbit anti-ASK1, Santa Cruz Biotechnology) were incubated in a humidified chamber at 37°C for 90 minutes, and the secondary antibodies (affinity-purified fluorescein isothiocyanate-conjugated anti-goat and tetramethyl-rhodamine isothiocyanate-conjugated anti-rabbit IgG; Molecular Probes, Eugene, OR) were incubated for 60 minutes. Confocal immunofluorescence microscopy was performed using the Olympus fluorescence/DIC microscope.

Adult Cardiomyocyte Contraction by Ca^{2+} Transient Recordings and Shortening Measurement

The detailed procedure for recording of Ca^{2+} transients in adult ventricular myocytes has been described in our previous publications.³⁴ Briefly, isolated rat adult myocytes were loaded for 15 minutes at room temperature with 1 $\mu\text{mol/L}$ of the fluorescent Ca^{2+} indicator fura 2-AM (Molecular Probes) in HEPES-buffered solution containing (mmol/L) KCl 5, NaCl 140, CaCl_2 2, MgCl_2 1, glucose 10, and HEPES 10 (pH 7.4). The coverslip with dye-loaded cells was mounted in a tissue chamber (Bellco) on the stage of a Nikon Diaphot inverted microscope equipped for epifluorescence (Deltascan 1, Photon Technology International). The myocytes were electrically stimulated at a frequency of 1 Hz with an S8 Grass stimulator. The cell was sequentially stimulated at 340- and 380-nm wavelength light using two excitation monochromators at a switching frequency of 100 Hz controlled by an optical chopper. The data collection rate at this switching frequency of 100 Hz was sufficient to resolve the rising and falling phases of the Ca^{2+} transient.

For measurement of shortening, each cell was stimulated with individual pulses (5V, 20 ms) by an isolated pulse stimulator (model 2100; A-M Systems, Carlsborg, WA) using an extracellular pipette as previously described.³⁵ Shortening was followed using a video edge detection system (VED-114; Crystal Biotech). A total of six cells from each group was measured. The degree of shortening (as expressed as a percentage of resting cell length) and the kinetics of shortening, time to peak shortening (t_{peak}), and time from peak to relaxation (t_{relax}) were recorded.

Statistical Analysis

Values are expressed as mean $\pm$ SD. A Student's *t*-test was used to evaluate differences in cardiomyocyte contraction. A level of $P < 0.05$ was accepted as statistically significant.

Figure 1. ASK1 specifically interacts with cTnT *in vitro*. **A:** A schematic diagram of ASK1 domains. ASK1-N comprises amino acids 1 to 678, ASK1-K comprises amino acids 678 to 936, ASK1-ΔN comprises amino acids 678 to 1375. **B:** Expression of ASK1-ΔN in yeast. Yeast cells harboring ASK1-ΔN expression plasmid or pAS2.1 (VC) were used for Western blot with anti-Gal4 antibody. **C:** Interaction of ASK1-ΔN with cTnT. Yeast transformants were grown on QDO (Ade⁻, Leu⁻, Trp⁻, His⁻) plates followed by the β -galactosidase filter assay. Interaction of two proteins (ASK1-ΔN and cTnT) permitted yeast to grow on QDO plate and became positive in β -galactosidase activity. Control vector (VC) with cTnT did not grow. **D** and **E:** Association of cTnT with the C-terminal domain of ASK1. HA-ASK1-WT, Flag-ASK1-ΔN, ASK1-N, or ASK1-K was transiently transfected in 293T cells. Association of ASK1 proteins with cTnT was determined by an *in vitro* binding to GST-cTnT followed by Western blot with either anti-HA (ASK1-WT) or anti-Flag (for ASK1-ΔN, ASK1-N, and ASK1-K). GST was used as a control.

Results

ASK1 Binds to cTnT in Yeast Two-Hybrid System and in Vitro

To identify substrates of ASK1 in cardiomyocytes, we used the constitutively active ASK1 (ASK1-ΔN, Figure 1A) as bait in the yeast two-hybrid system. Among 2×10^6 transformants screened from a human cardiac library, 10 clones were positive for growth on four-dropout medium (QDO, ade⁻, leu⁻, trp⁻, and his⁻) and for β -galactosidase assay. Sequence analysis revealed that five of the isolated cDNAs encoded the C-terminal domain (amino acids 120 to 288) of human cTnT (accession no. NM_000364, TNNT2 gene, human cardiac troponin T2). Further experiments using yeast two-hybrid system showed that co-transformation of ASK1-ΔN (Figure 1B), but not the empty vector pAS2.1 (VC), with cTnT was positive for growth on QDO and for β -galactosidase activity assay, confirming that cTnT specifically interacted with ASK1-ΔN (Figure 1C).

To identify the region of ASK1 associating with cTnT, we constructed expression constructs for various ASK1 domains as shown in Figure 1A—wild-type ASK1 (ASK1-WT, amino acids 1 to 1375), the N-terminal domain (ASK1-N, amino acids 1 to 678), the kinase domain (ASK1-K, amino acids 678 to 936) and the C-terminal domain (ASK1-ΔN, amino acids 678 to 1375). ASK1 expression constructs were transiently transfected in 293T cells. Association between cTnT and different domains of ASK1 was determined using an *in vitro* GST pull-down assay. Results show that GST-cTnT, but not GST alone,

Figure 2. ASK1 interacts with cTnT *in vivo*. **A:** Expression of cTnT and ASK1. 293T cells were transiently transfected with Flag-cTnT and HA-ASK1 expression plasmids as indicated. Expression of cTnT and ASK1 was examined by Western blot with anti-Flag and anti-HA, respectively. **B** and **C:** Association of cTnT with ASK1 *in vivo*. Association of cTnT with ASK1 was determined by immunoprecipitation (IP) with anti-Flag antibody followed by immunoblot (IB) with anti-HA (**B**). Association of cTnT with ASK1 was also determined by IP with anti-HA antibody followed by IB with anti-Flag (**C**).

interacted with HA-tagged ASK1-WT (Figure 1D). Using Flag-tagged ASK1 constructs, we found that GST-cTnT interacted with ASK1-ΔN, but not with ASK1-N or ASK1-K (Figure 1E). These data indicate that the C-terminal domain of ASK1 is responsible for cTnT association. ASK1 did not associate with cTnT by GST pull-down assay (data not shown).

ASK1 Associates with cTnT in Cardiomyocytes

To confirm the interaction of cTnT and ASK1 in intact cells, Flag-cTnT and HA-ASK1 expression plasmids were co-transfected in 293T cells. Expression of cTnT and ASK1 was determined by Western blot with anti-Flag and anti-HA, respectively (Figure 2A). The association between these two proteins was analyzed by co-immuno-

Figure 3. ASK1 associates with cTnT in adult cardiomyocytes. Rat adult cardiomyocytes were isolated and cultured as described. **A** and **B**: Association of endogenous ASK1 and cTnT was determined by IP with anti-cTnT (Santa Cruz) followed by IB with anti-ASK1 (H300, Santa Cruz) (**A**) or by IP with anti-ASK1 followed by IB with anti-cTnT (**B**). Normal rabbit serum (NS) was used as a control. **C**: Co-localization of ASK1 with cTnT in cardiomyocyte sarcomere. Localization of ASK1 and cTnT in rat adult cardiomyocyte was examined by indirect immunofluorescence confocal microscopy: Staining was performed with anti-ASK1 (followed by tetramethyl-rhodamine isothiocyanate-conjugated anti-rabbit IgG, **left**) and anti-cTnT (followed by fluorescein isothiocyanate-conjugated anti-goat IgG, **middle**). Merging picture is shown on the **right**.

precipitation (IP) assay. Figure 2B shows that IP with anti-Flag (cTnT) followed by Western blot with anti-HA precipitated ASK1-WT (Figure 2B). Figure 2C shows that IP with anti-HA (ASK1-WT) followed by Western blot with anti-Flag precipitated cTnT. The ability of an antibody against either Flag or HA to precipitate a complex that contains cTnT and ASK1 suggests that cTnT and ASK1 interact *in vivo*. To examine interaction of endogenous cTnT and ASK1, rat adult cardiomyocytes were isolated and cell lysates were used for IP assay. An antibody against cTnT, but not normal rabbit serum, specifically immunoprecipitated ASK1 (Figure 3A, lane 2). Conversely, an antibody against ASK1, but not normal rabbit serum, specifically immunoprecipitated cTnT (Figure 3B, lane 3), suggesting that endogenous ASK1 and cTnT form a complex in cardiomyocytes. We further examined cTnT-ASK1 interaction in adult cardiomyocytes by confocal microscopy. Localization of ASK1 and cTnT proteins was determined by indirect immunofluorescence confocal microscopy with anti-ASK1 (followed by tetramethyl-rhodamine isothiocyanate-conjugated anti-rabbit) and anti-cTnT (followed by fluorescein isothiocyanate-conjugated anti-goat). Results show that cTnT staining revealed an orderly sarcomere structure and ASK1 is co-localized with cTnT in sarcomere (Figure 3C). Treatment of H_2O_2 (100 μ mol/L for 30 minutes) did not alter the co-localization pattern (not shown). These data suggest that endogenous ASK and cTnT constitutively form a complex in adult cardiomyocytes.

ASK1 Phosphorylates cTnT at T194 and S198

cTnT is a component of the myofibrillar apparatus that is involved in Ca^{2+} -dependent regulation of contraction in

Figure 4. ASK1 phosphorylates cTnT at a consensus motif of ASK1 phosphorylation sites (T194/S198). **A** and **B**: ASK1 phosphorylates cTnT *in vitro*. **A**: Detection of the purified cardiac troponin proteins (Research Diagnostics). Ten μ g of cTn protein complex was run on 10% SDS-PAGE followed by Coomassie staining. cTnT (39 kD), cTnC (29 kD), and cTnI (18 kD) are indicated. **B**: cTnT, but not cTnC or cTnI, is phosphorylated by ASK1-ΔN. ASK1-ΔN protein was IP from ASK1-ΔN-expressing 293T cells with anti-Flag and normal serum (NS) was used as a control. Phosphorylation of cTn proteins by the ASK1-ΔN immunoprecipitate was examined by an *in vitro* kinase assay using the cTn complex as a substrate (+, lanes 2 and 4). No cTn complex was used as controls (-, lanes 1 and 3). Autoradiogram shown is representative of two similar experiments. **C** and **D**: Mutation at T194/S198 sites significantly reduced cTnT phosphorylation by ASK1-ΔN *in vivo*. **C**: Flag-tagged expression plasmids for ASK1-ΔN and the wild-type cTnT or mutant cTnT (cTnT-TS/AA) were co-transfected in 293T. Protein expression of cTnT and ASK1 was determined by Western blot with anti-Flag. **D**: Phosphorylation of cTnT by ASK1-ΔN was determined by IP with anti-Flag followed by an *in vitro* kinase assay. Empty vector (VC) was used as a control for ASK1-ΔN expression vector. Autoradiogram shown is representative of two similar experiments. Ratio of phosphorylated cTnT to expressed cTnT (p-cTnT/cTnT) was quantitated by densitometry. Data presented in the text are the average of two experiments.

cardiac muscles. Phosphorylation of myofibrillar proteins (including cTnT and cTnI) is important in the regulation of contractile activity.^{16,19,36} We examined if association of ASK1 with cTnT results in phosphorylation of cTnT. First we examined phosphorylation of cTnT by ASK1 *in vitro*. Purified human native cTnT (39 kD) (Figure 4A) present in a complex with cTnI (29 kD) and cTnC (18 kD) was incubated with Flag-tagged ASK1-ΔN protein IP with anti-Flag from ASK1-ΔN-expressing 293T cell lysates. Phosphorylation of cTn proteins by ASK1-ΔN was determined using an *in vitro* kinase assay. Phosphorylated cTnT was visualized by SDS/PAGE followed by autoradiography (Figure 4B). The identity of cTn proteins was determined by SDS-PAGE followed by Coomassie staining (Figure 4A). Results showed that cTnT, but not cTnI or cTnC, was specifically phosphorylated by immunoprecipitated ASK1-ΔN with anti-Flag (Figure 4B, lane 4). Consistent with findings by others,³⁷ ASK1-ΔN was autophosphorylated in this assay (Figure 4B, lanes 3 and 4). Cell lysates immunoprecipitated with normal sera showed no phosphorylation of cTnT or ASK1-ΔN (Figure 4B, lane 2).

ASK1 phosphorylates its substrates at a consensus sequence consisting of S/TxxS/T. There is only one ASK1 phosphorylation consensus sequence in cTnT (T¹⁹⁴ERKS¹⁹⁸). To determine whether ASK1 phosphorylates cTnT molecule at this site, we constructed a cTnT

Figure 5. ROS induces activation of ASK1, cTnT phosphorylation, and inhibition of contractility in cardiomyocytes. Adult rat cardiomyocytes were cultured in 6-well plates and were either untreated (Ctrl) or treated H₂O₂ (100 μ mol/L for 1, 5, or 15 minutes). **A:** H₂O₂ induces activation of ASK1. ASK1 activity was measured by IP with anti-ASK1 followed by an *in vitro* kinase assay using GST-MKK4 as a substrate. Autoradiogram shown is representative of three similar experiments. **B:** H₂O₂ induces cTnT phosphorylation. Cells were labeled with ³²P-orthophosphate as described. cTnT protein was IP by anti-cTnT and phosphorylation of cTnT was examined by SDS/PAGE. Autoradiogram shown is representative of two similar experiments.

mutant with T194A and S198A (cTnT-TS/AA) in a mammalian expression Flag-vector. Flag-tagged wild-type cTnT or cTnT-TS/AA were co-expressed with Flag-tagged ASK1- Δ N in 293T cells and expression was determined by Western blot with anti-Flag (Figure 4C). Phosphorylation of cTnT was determined by immunoprecipitation with anti-Flag followed by an *in vitro* kinase assay in the presence of γ -³²P-ATP (Figure 4D). Phosphorylation of cTnT-TS/AA was reduced compared to wild-type (lane 2 versus lane 4 in Figure 4D). Densitometry analyses showed that mutations at the ASK1 phosphorylation consensus site reduced the extent of cTnT phosphorylation by ~50%. These data indicate that T¹⁹⁴ERKS¹⁹⁸ of cTnT are the major phosphorylation sites for ASK1. The presence of residual phosphorylation in cTnT-TS/AA suggests that a second site in cTnT is phosphorylated by ASK1 or ASK1-associated kinase(s) in the immunoprecipitates.

ROS Induces ASK1 Activation and cTnT Phosphorylation in Rat Adult Cardiomyocytes

There is increasing support for the idea that excessive production of proinflammatory mediators such as TNF and ROS contribute to the pathogenesis of cardiac dysfunction.^{1–6} To determine whether ROS activates ASK1 in cardiomyocytes, rat adult cardiomyocytes were treated with H₂O₂ (100 μ mol/L) for 1, 5, or 15 minutes. ASK1 activity was measured by an *in vitro* kinase assay using GST-MKK4 as a substrate.^{28,29} Phosphorylation of endogenous cTnT in cardiomyocytes was examined by *in vivo* labeling with ³²P-orthophosphate and cell lysates were immunoprecipitated with anti-cTnT followed by SDS-PAGE autoradiography. Consistent with data from endothelial cells,^{28,29} H₂O₂ induced ASK1 activation in cardiomyocytes at 1 minute with a peak at 5 minutes (Figure 5A). ASK1 activity was associated with a concomitant increase in cTnT phosphorylation with peak at 5 minutes (Figure 5B). There are two major phosphorylated bands. As shown by studies from Saggin and colleagues,³⁸ the rat heart has two cTnT forms—the 42.5 kd in fetal ventricles and the 41 kd in adult ventricles. Based on the molecular weight (one is ~41 kd and another ~30 kd, Figure 5B), the top band is cTnT and the lower one is either degraded cTnT or cTnI

(~30 kd), which associated with cTnT in the immunoprecipitates. This explains why the lower band is weaker than the top band (cTnT).

ASK1 Expression Induces cTnT Phosphorylation and Contractile Dysfunction in Rat Adult Cardiomyocytes

To establish a connection between ASK1 activation, cTnT phosphorylation, and cardiac contractile dysfunction, we intend to generate transgenic mice that have cardiac-specific expression of ASK1. We found that the cardiac-specific expression (driven by the myosin heavy chain promoter) of a constitutively active form (ASK1- Δ N), but not an inactive form of ASK1 (ASK1-N),^{28,29} caused embryonic lethality in transgenic mice (Min et al, unpublished data). Thus we cannot examine effects of ASK1 on cTnT *in vivo*. Therefore we have examined effects of ASK1 expression on cTnT phosphorylation and cardiac contractility in isolated rat adult cardiomyocytes. We constructed a lentiviral expression vector co-expressing ASK1- Δ N and GFP on a bi-cis-tronic mRNA (HR-ASK1- Δ N-GFP) and the lentiviruses were prepared to a titer of 10⁷ pfu/ml. Rat adult cardiomyocytes were infected with HR-ASK1- Δ N-GFP or empty vector HR-GFP (VC). Infection of cardiomyocytes by the lentiviral system was confirmed by visualizing GFP under microscope. Results show that cardiomyocytes were effectively infected by the lentiviral system (100% infection with MOI of 50). Expression of ASK1- Δ N was determined by Western blot with anti-Flag (Figure 6A) and ASK1 activity was determined by an *in vitro* kinase assay using GST-MKK4 as a substrate (Figure 6A). Phosphorylation of endogenous cTnT in cardiomyocytes was also examined by *in vivo* labeling with ³²P-orthophosphate as described above. Results show that ASK1- Δ N expression induced significant amount of cTnT phosphorylation (Figure 6A). We then examined the effect of ASK1 activation on cardiomyocyte shortening. Adult cardiac myocytes were stimulated, shortening amplitudes and kinetics were measured as described. The myocytes overexpressing ASK1- Δ N were rod shaped and looked healthy under microscope, indicating that ASK1 does not induce apoptosis of cultured adult cardiomyocytes. The degree of shortening (as expressed as a percentage of resting cell length) and the kinetics of shortening, time to peak shortening (t_{peak}) and time from peak to relaxation (t_{relax}), were recorded. Lentiviral infection or ASK1 activation had no effects on the resting cell length. However, ASK1 activation significantly reduced the degree of shortening (percentage of resting cell length from 17.27 micrometer in VC group to 8.29 micrometer in ASK1- Δ N group) (Figure 6B). In contrast, mean t_{peak} shortening (1.45 seconds in VC group compared to 2.11 seconds in ASK1- Δ N group) and t_{relax} relaxation (1.28 seconds in VC group compared to 1.55 seconds in ASK1- Δ N group) were significantly increased by ASK1 activation (Figure 6, C and D).

In cardiac cells, each contraction is dependent on a transient elevation of Ca²⁺. Thus, we recorded the frequency of fura-2-reported Ca²⁺ transients as a measure-

Figure 6. Overexpression of constitutively active ASK1 in cardiomyocytes induces cTnT phosphorylation, and reduces cardiomyocyte contractility. Rat adult cardiomyocytes were infected with either empty vector (VC) or ASK1-ΔN-expressing lentivirus at MOI of 50. Six to 12 hours after infection, ASK1-ΔN expression and activity, cTnT phosphorylation, MgATPase activity, and contractility were determined as described. **A:** ASK1-ΔN expression, activity, and cTnT phosphorylation in cardiomyocytes. Infection efficiency of cardiomyocytes by the HIV-derived lentiviral system was determined for GFP-positive cells under a fluorescence microscope. Expression of ASK1-ΔN by Western blot with anti-Flag (**top**). ASK1 activity was measured for ASK1 activity using the *in vitro* kinase assay using GST-MKK4 as a substrate (**middle**). Cells were labeled with 32 P-orthophosphate as described. cTnT protein was IP by anti-cTnT and phosphorylation of cTnT was examined by SDS/PAGE (**bottom**). Autoradiogram shown is representative of two similar experiments. **B to D:** Effects of ASK1 activation on cardiomyocyte shortening. Adult cardiac myocytes were field stimulated, shortening amplitudes and kinetics were measured as described. Shortening was followed using a video edge detection system. A total of six cells from each group were measured. The mean percentage of resting cell length, t_{peak} shortening, and t_{relax} relaxation are presented. **E:** Inhibition of contractility of cardiomyocyte by ASK1 by measuring Ca^{2+} transients. Control (VC) or ASK1-expressing rat adult cardiomyocytes (ASK1-ΔN) were field stimulated at 1 Hz and fura-2-reported Ca^{2+} transients were measured as described in Experimental Procedures. Data presented as fura-2 340/380-nm fluorescence ratio calibrated in terms of cytosolic-free Ca^{2+} .

ment of adult cardiomyocyte contraction. To determine effect of ASK1 expression on cardiac contractility, ASK1-expressing adult cardiac myocytes were field-stimulated at 1 Hz and Ca^{2+} transients were measured as previously described.³⁴ Results show that ASK1 expression significantly inhibited both the rate and amplitude of Ca^{2+} transients (Figure 6E). These data strongly suggest that ASK1 activation induces cardiac contractile dysfunction.

Discussion

The major finding of the present study is that ASK1 directly associates with and specifically phosphorylates cardiac TnT. Moreover, ASK1 activation induces inhibition of cardiomyocyte contractility. Because ASK1 is critical for ROS/proinflammatory cytokine-induced signaling, our data suggest that ASK1 may participate in the patho-

genesis of cardiomyopathy and heart failure induced by ROS and cytokines.

It has been shown that phosphorylation of cTnT and cTnI diminishes contractile activity.¹⁹ Noland and Kuo¹⁹ showed that exclusive phosphorylation of cTnT results in ~60% decrease in maximum MgATPase activity in fully reconstituted systems. When cTnI was exclusively phosphorylated under the same conditions, only ~20% decrease was observed, suggesting that cTnT phosphorylation is important in regulating cardiac contractility. Although the kinases responsible for cTnI phosphorylation have been investigated,^{18–21,36} much less is known about cTnT. cTnI can be phosphorylated by at least three protein kinases, Ca^{2+} -phospholipid-dependent protein kinase (PKC), cGMP-dependent protein kinase (PKG), and cAMP-dependent kinase (protein kinase A). It has been shown that phosphorylation of cTnI by PKC at S43 and S45 is important for PKC-mediated regulation of actomyosin MgATPase. Phosphorylation of cTnI at S23 and S24 by protein kinase A or PKG results in a decrease in the sensitivity of the contractile apparatus to Ca^{2+} because of decreased affinity of phosphorylated cTnI to cTnC. Recently it has been reported that cTnT interacts with PKG and functions as an anchoring protein to mediate cTnI phosphorylation by PKG.³⁶

In the present study, we present data that ASK1 directly associates with and specifically phosphorylates cTnT (but not cTnI and cTnC). ASK1 phosphorylates cTnT at sites T194 and S198 within an ASK1 consensus phosphorylation sequence (although other sites may also be phosphorylated). Mutation of this consensus motif of ASK1 phosphorylation sites significantly reduced the extent of cTnT phosphorylation *in vivo*, indicating that ASK1 is a major kinase responsible for cTnT phosphorylation. This sequence is located at the C-terminus of cTnT, the region that is responsible for association with the C-terminus of cTnC, the N-terminus of cTnI, and the Ca^{2+} -dependent interaction with tropomyosin. It is conceivable that interaction with ASK1 and subsequent phosphorylation by ASK1 may significantly alter interactions of cTnT with other contractile components leading to reduction of MgATPase and contractile dysfunction.

Mutation of the consensus motif of ASK1 phosphorylation sites did not completely abolish cTnT phosphorylation, suggesting presence of other sites in cTnT that are phosphorylated by ASK1 (or an ASK1-associating kinase present in the immunoprecipitate). It was reported that three putative PKC phosphorylation sites (T194, T203, and T284 of the human sequence) are located at the C-terminus of cTnT. Recently, it has been shown that a transgenic mouse expressing skeletal TnT that naturally lacks these PKC sites present in cTnT shows blunted PKC-mediated depression of contraction, implying the importance of these PKC phosphorylation sites in PKC regulation of cardiac contractility.³⁹ It is plausible that ASK1 and PKC cooperatively mediate ROS-induced cardiac contractile dysfunction. The data that both ASK1 and PKC are activated by ROS and ROS induces more profound effects than ASK1 expression on cardiomyocyte contractility support this model.

Our data also show that ASK1 has an inhibitory effect on shortening and Ca^{2+} transient in both contraction and relaxation. It is possible that ASK1 may have multiple targets including Ca^{2+} handling proteins such as phospholamban, the sarcolemmal Ca^{2+} channel and the SR ryanodine receptor (RyR). It has been proposed that PKC targets on both cTnT and cTnI to synergistically induce inhibition on the actin-myosin interaction.³⁹ It is conceivable that ASK1 targets on multiple contractile components to exert its inhibitory effects on cardiac contractility. Consistently, JNK, a downstream kinase of ASK1, has been shown to induce down-regulation of gap junction connexin-43 and impaired intracellular communication in the failing heart.⁴⁰ TAK1 (another member of MAP3K family) induces heart failure by inducing cardiac hypertrophy, fetal gene expression, and apoptosis.⁴¹

ASK1 has been implicated in the intracellular signaling cascades triggered by stress stimuli.^{22–26} A specific role of ASK1 in TNF and ROS signaling is supported by recent data from the ASK1-deficient mice.²³ Our data show that overexpression of ASK1 induces cTnT phosphorylation and inhibits contractility in cardiomyocytes, demonstrating a novel function of ASK1 in regulating cardiac function. This role is supported by our recent observation that transgenic mice in which the constitutively active ASK1 is expressed in the heart (α -myosin heavy chain promoter) die before birth (Min et al, unpublished data). It has not been determined whether lethality of ASK1-transgenic mice is because of ASK1-induced contractile dysfunction or apoptosis. However, these two events may be functionally linked in ROS/cytokine-induced cardiac pathogenesis.⁴² Our data show that ASK1 does not induce apoptosis of cultured cardiomyocytes. Consistent with our observation, it has been recently shown that ASK1 regulates cardiomyocyte hypertrophy by activating nuclear factor- κ B.⁴³

In summary, these data define a role for ASK1 in ROS-induced cardiac contractile dysfunction. Although the roles of ASK1 in heart function have been studied to a limited extent, this study suggests that inhibiting ASK1 activity may provide a valid approach for treatment of cardiovascular diseases.

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