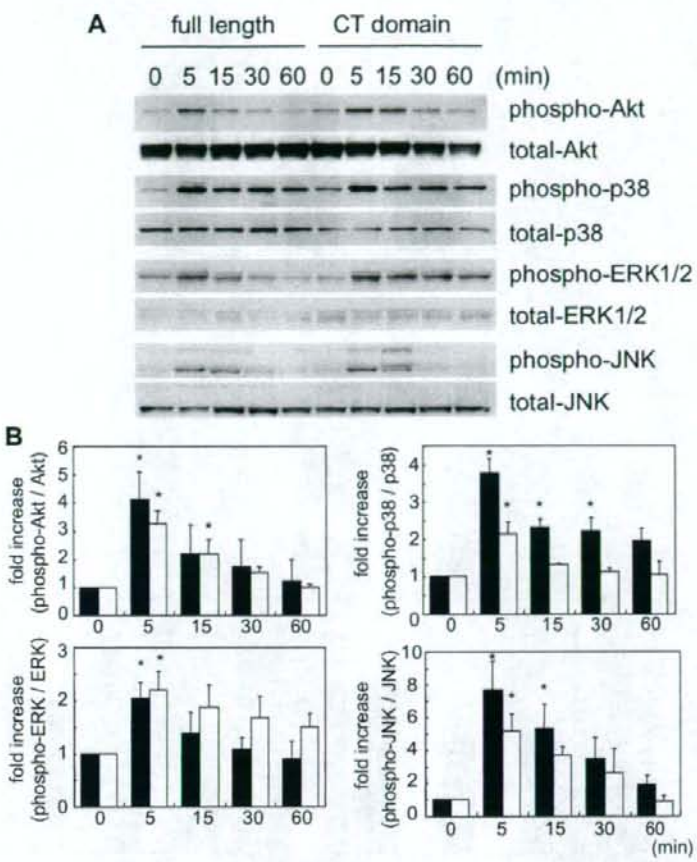

Fig. 2. CTGF activates Akt, p38 MAPK, ERK1/2, and JNK in cardiomyocytes. (A) Cardiomyocytes were stimulated with 1.3 nM full length or CT domain of CTGF for the indicated time. Cell lysates were immunoblotted with anti-phospho-Akt, anti-phospho-p38 MAPK, anti-phospho-ERK1/2, or anti-phospho-JNK antibody. Blots were reprobed with anti-Akt, anti-p38 MAPK, anti-ERK1/2, or anti-JNK antibody. Representative data are shown. (B) Quantitative analysis of phosphorylation of Akt, p38 MAPK, ERK1/2, and JNK. The band intensities of phosphoproteins were normalized with those of total proteins. Black and white bars represent full length and CT domain of CTGF, respectively. Data are presented as the means ± SE ($n = 3$). $^{*}P < 0.05$ versus 0 min.

CTGF did not induce the phenotypic changes in the gene expression profile

GPCR ligands, such as ET-1 and noradrenalin, induces cardiomyocyte hypertrophy, accompanied by the changes in gene expression profile. Therefore, we investigated whether CTGF treatment leads to the phenotypic changes. Northern-blot analyses have demonstrated that both skeletal actin and BNP mRNA were upregulated by ET-1, while CTGF exhibited no effects on the expression of these genes (Fig. 3B). These data suggest that CTGF mediated hypertrophic signals though the distinct pathway from ET-1.

Activation of Akt is required for CTGF-mediated increase in cell size

So far, it has been reported that the activation of MAPK family is a critical event in the onset of cardiac hypertrophy. Therefore, we examined the importance of ERK1/2, p38 MAPK, or JNK in CTGF-mediated increase in cell size by using their specific inhibitors, U0126, SB202190, or SP600125. As shown in Fig. 4A, CTGF-induced increase in cell size was not affected by these inhibitors, suggesting that MAPK family pathways are unlikely to be necessary for cardiomyocyte hypertrophy in CTGF signaling.

Recent studies have demonstrated that Akt plays important roles in the determination of cell size both *in vitro* and *in vivo* [17]. Therefore, we examined the effects of the inhibition of Akt by using adenovirus vector expressing dominant-negative Akt (dnAkt). Adenoviral transfer of dnAkt inhibited CTGF-mediated cardiomyocyte hypertrophy, while the transfection of adenovirus vetor expressing β-gal showed no effects (Fig. 4B).

Discussion

In the present study, we demonstrated that full length of recombinant human CTGF induced cardiac hypertrophy in cultured cardiomyocytes. The C-terminal region of CTGF exhibited the similar effects in cardiac myocytes. However, neither skeletal actin nor BNP mRNA was upregulated by CTGF, unlike GPCR ligands such as ET-1. CTGF rapidly activated p38 MAPK, ERK1/2, JNK, and Akt. Inhibition of Akt by adenoviral transduction of

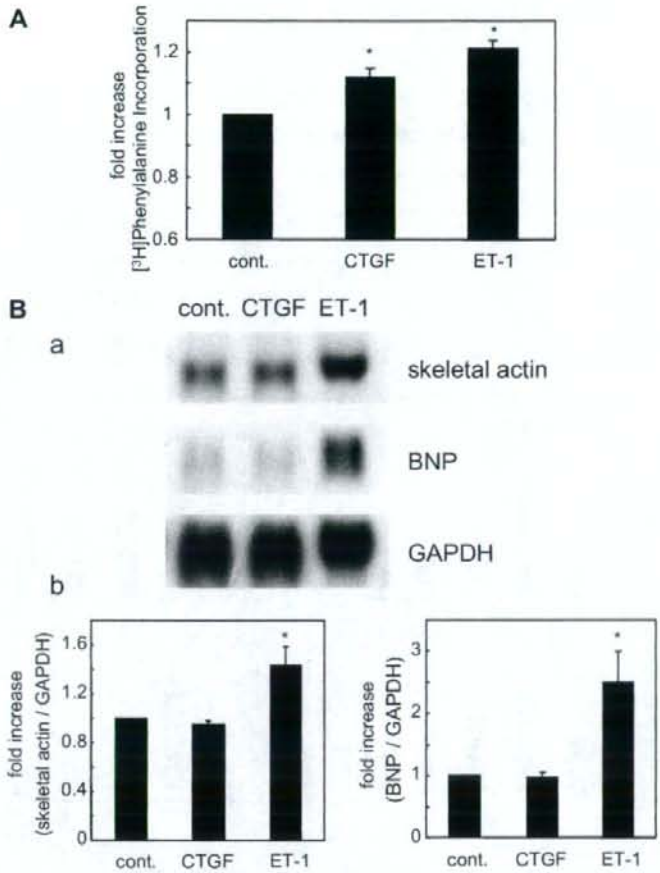

Fig. 3. Characterization of CTGF-induced cardiomyocyte hypertrophy. (A) Cardiomyocytes were stimulated by 1.3 nM full length of CTGF or 10 nM endothelin-1 (ET-1) in the presence of [^{3}H] phenylalanine for 48 h. The incorporation of the radioactivity of [^{3}H] phenylalanine was measured. Data are presented as the means ± SE ($n = 9$). $^*P < 0.05$ versus control. (B) Cardiomyocytes were stimulated by 1.3 nM full length of CTGF or 10 nM ET-1 for 48 h. Total RNA was Northern-blotted for skeletal actin, BNP, and GAPDH. (a) Representative data are shown. (b) The band intensities of skeletal actin and BNP were normalized with GAPDH. Data are presented as the means ± SE ($n = 3$). $^*P < 0.05$ versus control.

dnAkt ameliorated CTGF-mediated cardiomyocyte hypertrophy. These data suggest that CTGF induces cardiomyocyte hypertrophy through Akt pathway.

CTGF is a member of CCN family proteins which contain four modules in the structure, ie, insulin-like growth factor (IGF)-binding protein module (IGF-BP), von Willebrand factor type-C (VWC), thrombospondin type-1 (TPS-1), and C-terminal module (CT). As IGF-1 is a well-known hypertrophic growth factor[18], we addressed whether CTGF mediates cardiac hypertrophy through its interaction with IGF-1. Since IGF-1-binding domain is located at N-terminal region of CTGF molecule, we examined the hypertrophic effects of CT. As is the case with full length CTGF, CT induced the cardiomyocyte hypertrophy in a dose dependent manner. Moreover, maximal effect of CT is almost equivalent to that of full length CTGF. These findings suggest that the interaction with IGF-1 is not required for CTGF-mediated cardiac hypertrophy and that the hypertrophic signal of CTGF is mediated mainly through its C-terminal region, though we cannot exclude the possibility that the other regions modulate some phenotypes in hypertrophied cells.

CTGF expression is upregulated by the hypertrophic agonists for G-protein coupled receptors (GPCRs), including endothelin-1 (ET-1), and phenylephrine [4]; however, it should be noted that CTGF induces cardiomyocyte hypertrophy that is distinct from the phenotype seen after the stimulation with GPCR ligands. Accompanied by hypertrophic changes, skeletal actin and BNP were induced by ET-1, while not by CTGF. Consistently, CTGF utilizes the distinct signaling pathways from GPCR ligands in the induction of hypertrophy. The inhibition of Akt pathway by dnAkt abrogated CTGF-mediated increase in cardiomyocyte size, while the pharmacological inhibition of MAPK pathways, which transduces hypertrophic signals in response to GPCR ligands [19], did not influence cardiomyocyte size. Collectively, Akt activation is required for CTGF-mediated induction of hypertrophy. Interestingly, recent studies have demonstrated that Akt plays an important role in the determination of the cell size in cardiac myocytes [20].

Though pro-fibrotic effects of CTGF have been reported [21], further efforts are required to elucidate pathophysiological significances of CTGF-mediated hypertrophy. Recent studies have demonstrated that the transient activation of Akt in cardiac myocytes

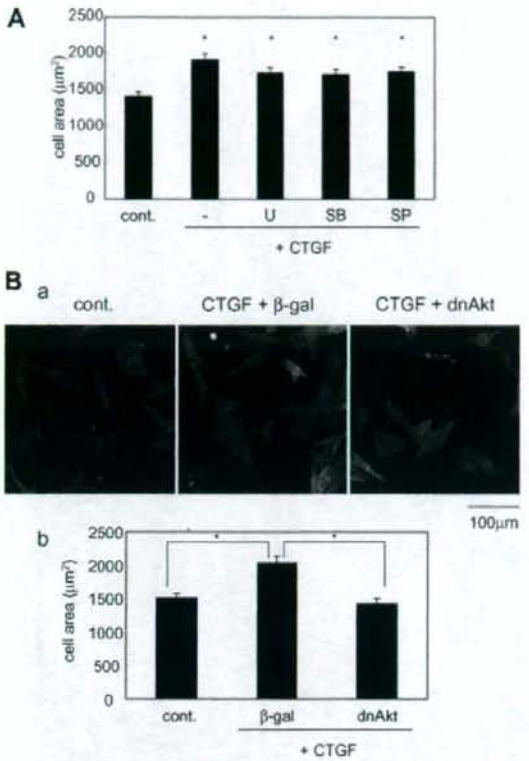

Fig. 4. Dominant negative Akt inhibits CTGF-induced cardiac hypertrophy. (A) Cardiomyocytes were subjected to full length of 1.3 nM CTGF with 5 μM U0126, 5 μM SB202190 or 1 μM SP600125, respectively, and the cell size was quantitatively analyzed as described in Fig. 1. Data are shown as the means ± SE, $^{*}P < 0.05$ versus control. The experiments were repeated three times with similar results. (B-a) Cardiomyocytes were subjected to full length of 1.3 nM CTGF with β-gal or dnAkt. Representative immunofluorescent micrographs are shown. (B-b) The cell size of cardiomyocytes was quantitatively analyzed. The cell surface area was estimated as described in Fig. 1. Data are shown as the means ± SE, $^{*}P < 0.001$. The experiments were repeated three times with similar results.

results in reversible cardiac hypertrophy with normal cardiac function, while its continuous activation leads to severe cardiac dysfunction, accompanied by fibrosis [22]. CTGF might function differentially according to the pathological stages.

In summary, we have demonstrated that CTGF induced the increase in cell size through Akt pathway in cardiomyocytes. This is the first characterization of CTGF as a hypertrophic factor in cardiac myocytes.

Acknowledgments

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Degradation of NFAT5, a Transcriptional Regulator of Osmotic Stress-related Genes, Is a Critical Event for Doxorubicin-induced Cytotoxicity in Cardiac Myocytes*

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Nuclear factor-activated T cell 5 (NFAT5), a novel member of the NFAT family of proteins, was originally identified as a transcriptional factor responsible for adaptation to hyperosmotic stress. Though NFAT5 is ubiquitously expressed, the biological functions of NFAT5 remain to be clarified, especially in the tissues that are not exposed to hypertonicity, including hearts. In the present study, we focused on the cardioprotective roles of NFAT5 against the cardiotoxic anti-tumor agent doxorubicin (Dox). In cultured cardiomyocytes, transcripts of the hypertonicity-inducible genes, such as taurine transporter (*TauT*) and sodium/myo-inositol transporter, were down-regulated by Dox. Interestingly, NFAT5 protein, but not mRNA, was decreased in cardiomyocytes exposed to Dox. Treatment of proteasome inhibitors, MG-132 or proteasome-specific inhibitor 1, prevented the Dox-mediated decrease of NFAT5 protein. Further, ubiquitin-conjugated NFAT5 was not detected in cultured cardiomyocytes treated with MG-132 and/or Dox, as assessed by immunoprecipitation assay, suggesting Dox-induced degradation through ubiquitin-independent proteasome pathway. Importantly, inhibition of NFAT5 with overexpression of dominant-negative NFAT5 decreased cell viability and increased creatine kinase leakage into culture medium. Consistently, small interfering RNA targeting NFAT5 gene enhanced myocyte death. These findings suggest that Dox promoted the degradation of NFAT5 protein, reducing cell viability in cardiomyocytes. This is the first demonstration that NFAT5 is a positive regulator of cardiomyocyte survival.

Because mature cardiac myocytes have a limited proliferative potential, accumulation of cardiomyocyte death leads to heart failure. Pathologically, cardiac myocyte death is induced by various kinds of stresses, such as hypoxia, mechanical stress, and cardiotoxic drugs. Among them, doxorubicin (Dox),[2] an anti-tumor agent of the anthracycline family, is well known to have a harmful effect and its use is limited by irreversible cardiotoxicity (1, 2). Although the precise mechanisms of its myocardial damage are unclear, numerous evidence suggests that Dox induces cardiotoxicity through multiple pathways, including production of reactive oxygen species, perturbation of calcium handling, and selective inhibition of cardiac muscle-specific gene expression (1, 3, 4). Recently, several reports indicate that Dox activates proteasome-mediated proteolysis that results in the disorder of cardiac gene expression (5, 6).

NFAT5 (nuclear factor-activated T cell 5)/TonEBP (tonicity-response element-binding protein), a member of the rel/NFκB/NFAT family of transcription factors, was originally identified as a transcriptional factor involved in the cellular responses to hypertonic stress (7, 8). Whereas NFAT 1–4 are activated by Ca^{2+}/calcineurin pathway, NFAT5 activity is regulated in a calcineurin-independent manner, because it lacks the N-terminal NFAT homology region containing the calcineurin regulatory motif (7, 8). NFAT5 is activated by phosphorylation under a hyperosmotic environment (7, 8). NFAT5 transcriptionally regulates the expression of target genes responsible for the metabolism of organic osmolytes, including aldose reductase (9), taurine transporter (TauT) (10), betaine/GABA transporter (BGT-1) (11), and sodium/myo-inositol transporter (12, 13). NFAT5 also induces molecular chaperones, such as heat shock protein 70-2 (Hsp70-2) (14) and osmotic stress protein of 94 kDa (Osp94) (15). Importantly, inhibition of NFAT5 led to an increase in susceptibility to hypertonic stress (16).

NFAT5 is ubiquitously expressed even in tissues that are not exposed to hypertonic environment, such as brain, heart, and skeletal muscle (17, 18). Recent studies have demonstrated that disruption of *NFAT5* gene results in late gestational lethality and that surviving *NFAT5*$^{-/-}$ mice develop a remarkable atrophy of kidney medulla (19). Similarly, suppression of NFAT5 transcriptional activity resulted in impaired cell proliferation with increased cell death in T-cell (16) and lens fiber cells (20). Interestingly, NFAT5, unlike the other members of the NFAT family of protein, is localized not only in cytosol but also in

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[2] The abbreviations used are: Dox, doxorubicin; NFAT5, nuclear factor-activated T cell isoform 5; dnNFAT5, dominant-negative form of NFAT5; TauT, taurine transporter; BGT-1, betaine/γ-butyric acid (GABA) transporter-1; SMIT, sodium/myo-inositol transporter; Hsp70-2, heat shock protein 70-2; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CPK, creatine phosphor-kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; HEK, human embryonic kidney; EMSA, electrophoretic mobility shift assay.

nucleus in isotonic conditions (9). Taken together, it is proposed that NFAT5 activity is positively or negatively regulated independently of tonicity during normal development, although the tonicity-independent mechanisms for NFAT5 activation remain to be fully elucidated.

In the present study, we demonstrated that transcriptional activity of NFAT5 was suppressed by Dox exposure in cultured cardiomyocytes in a proteasome-dependent manner. Then we tested the cytoprotective effects of NFAT5 activity by using the dominant-negative form of NFAT5. This is the first demonstration that NFAT5 functions as an important mediator for cardiomyocyte survival.

EXPERIMENTAL PROCEDURES

Cell Culture—Cardiac myocytes were cultured as described previously (21, 22). Briefly, cardiac ventricles of 1-day-old Wistar Kyoto rats were minced and dissociated with 0.1% trypsin and 0.1% collagenase type IV. To eliminate the non-myocyte population, dispersed cells were plated in culture dishes and incubated for 1 h at 37 °C. Nonattached cells were collected as cardiomyocytes and cultured in Dulbecco's modified essential medium/Ham's F-12 (DMEM/F-12) containing 5% neonatal calf serum and 5-bromo-2′-deoxyuridine. On the other hand, attached cells were cultured in DMEM/F-12 containing 5% neonatal calf serum and used as cardiac fibroblasts after being passed twice.

After being cultured for 2 days, cells were washed twice and then treated with Dox (a kind gift from Kyowa Hakko Kogyo Co. Ltd.) in serum-free DMEM/F-12. Cycloheximide (5 μM; Nacalai Tesque), MG-132 (5 μM; Calbiochem), proteasome inhibitor I (10 nM; Peptide Institute), U0126 (Cell Signaling), SB202190 (Alexis), SP600125 or H-7 (Calbiochem) was used to pretreat cells for 1 h prior to Dox treatment.

Adenovirus infection was performed 24 h after seeding. Cells were cultured with adenovirus vector at a multiplicity of infection of 20. The human embryonic kidney cell line (HEK293 cells) was cultured in minimum essential medium containing 10% fetal bovine serum.

Northern Blot—Northern blot was performed as described previously (10). The cDNA fragments for NFAT5, TauT, SMIT, and glyceraldehyde-3-phosphate dehydrogenase were labeled with [α-^{32}P]dCTP (PerkinElmer Life Sciences) using the Mega-Prime DNA labeling system (Amersham Biosciences).

Western Blot—Western blot was performed as described previously (10). Anti-NFAT5 (H-300), anti-ubiquitin (P4D1) (Santa Cruz Biotechnology), anti-NFAT5 (1439–1455) (Oncogene), anti-phospho extracellular signal-regulated kinase (ERK), anti-phospho p38 MAPK, anti-phospho c-Jun N-terminal kinase (JNK) (Cell Signaling), and anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon) were used.

Immunoprecipitation—Immunoprecipitation was performed using cell lysates from cultured cardiomyocytes. Protein samples in the lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0) containing 1% Nonidet P-40 and protease inhibitor mixture (Nacalai Tesque) were incubated on ice and rotated at 4 °C for 2 h with mouse anti-ubiquitin antibody (P4D1), and then protein G-conjugated Sepharose beads (Santa Cruz Biotechnology) were added to samples and rotated at 4 °C for 1 h. The beads were collected by centrifugation at 3,000 rpm for 1 min and washed three times with the lysis buffer without Nonidet P-40. Samples were boiled for 10 min in SDS buffer (25 mM Tris-HCl, pH 6.8, 0.8% SDS, 4% glycerol, 2% 2-mercaptoethanol) for Western blot analysis.

Immunofluorescence Microscopic Examination—Immunofluorescence microscopic examination was performed as described previously (23). Briefly, cardiomyocytes plated on the coverslip were fixed in phosphate-buffered saline containing 2% paraformaldehyde for 20 min, permeabilized in phosphate-buffered saline containing 0.3% Triton X-100 for 10 min, and blocked in phosphate-buffered saline containing 1% bovine serum albumin. Immunostaining was performed using anti-NFAT5(1439–1455) antibody (1:1000; Oncogene). Alexa Fluor 488 secondary antibody (Molecular Probes) was used for detection. Nuclei were stained with Hoechst 33258. Cells were examined by Olympus IX70.

Preparation of Nuclear Extracts—Nuclear extracts were prepared form cardiomyocytes or NFAT5-expressing HEK293 cells as described previously (24). Briefly, cells were washed three times in ice-cold phosphate-buffered saline and then scraped into lysis buffer (20 mM HEPES, pH 7.6, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Nonidet P-40, 10 mM NaCl, 1 mM dithiothreitol, protease inhibitor mixture (Nacalai Tesque)). Cells were lysed by incubation on ice for 10 min. Samples were centrifuged at 2,000 rpm for 10 min, and the supernatant fraction was discarded. The pellet was resuspended in cold nuclear extract buffer (20 mM HEPES, pH 7.6, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Nonidet P-40, 500 mM NaCl, 1 mM dithiothreitol, protease inhibitor mixture) and incubated on ice for 1 h. Cellular debris was removed by centrifugation at 10,000 rpm for 10 min at 4 °C, and the supernatant containing nuclear proteins was used for assay after protein concentrations were measured with BCA protein assay.

Luciferase Assay—Promoter-reporter plasmids pTauT/-862-Luc, pTauT/-124-Luc, pTauT/-99-Luc, and pTauT/-124m-Luc were constructed as described previously (10). Transient transfection into cardiac myocytes was performed by using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. Assay was performed by using the Dual Luciferase assay system (Promega) as described previously (10). Expression vector of truncated NFAT5 construct consists of a DNA-binding domain (212–543 amino acids) as described previously (10). Other investigators have revealed that the deletion mutant inhibits endogenous NFAT5 activity, interfering with NFAT5 dimerization but not the transcriptional activity of the other NFATs, NFκB and AP-1 (16, 25).

Protein Expression in HEK293 Cells—HEK293 cells seeded in 100-mm dishes were transfected with the NFAT5 expression vector pFLAG-NFAT5 (10) by using FuGENE 6 transfection regent (Roche Applied Science) and were then cultured for 48 h.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as described previously (24). To prepare DNA probes for EMSA, single-strand oligonucleotides (TauT-NFAT5 sense, AGC TGG TAT TTT TCC ACC CAG; TauT-NFAT5 antisense, CTG GGT GGA AAA ATA CCA GCT) were

end labeled by [γ-^{32}P]ATP and then were annealed at room temperature. Nuclear extracts were incubated with ^{32}P-labeled DNA probe and poly(dI-dC) (100 μg/ml) at 30 °C for 20 min. To perform the competition assay, excess concentration of wild-type or mutant-type double-strand oligonucleotides (TauT-NFAT5 mut sense, AGC TGA TCT TCC CTT ACC CAG; TauT-NFAT5 mut antisense, CTG GGT AAG GGA AGA TCA GCT; mutated nucleotides are underlined) or BGT-NFAT5 oligonucleotide (BGT-NFAT5 sense, ACC AGC GGT AAT TTT CCA CCC AG; BGT-NFAT5 antisense, CTT GGT GGA AAA TTA CCG CTG GT) was preincubated for 5 min, followed by incubation with radiolabeled probe. For supershift assay, 2 μg of antibodies (anti-NFAT5 antibody or control IgG) was used. The DNA-protein complex was fractionated by 5% polyacrylamide gel. The gels were dried and processed for autoradiography.

Generation of Recombinant Adenovirus and Adenovirus Infection into Cardiomyocytes—To construct the recombinant adenovirus vector encoding dominant-negative NFAT5 (dnNFAT5), the cDNA encoding the DNA-binding domain of NFAT5 was cloned into the cloning site of pACCMV.pLpA (26). Subsequently, the recombinant plasmid was cotransfected with pJM17 plasmid into HEK293 cells, and the replication-deficient adenovirus was generated via homologous recombination as described previously (21, 26).

Cardiac myocytes were infected with adenovirus in medium with 5% fetal calf serum at multiplicity of infection of 20 and cultured for 2 days. Adenovirus carrying β-galactosidase was used as a control. The infection efficiency of the adenovirus was almost 100% in cultured cardiomyocytes confirmed by immunocytochemistry (Fig. 7*A*).

Small Interfering RNA (siRNA) Studies—For siRNA analysis, TriFECTa Dicer-substrate RNA interference kit (Integrated DNA Technologies Inc.) containing 27-mer duplex was used. The following sequences specific for rat NFAT5 gene were used: sense 5′-GCC UCG ACC CUA GCA GCA ACA UGA C-3′, antisense 5′-GUC AUG UUG CUG CUA GGG UCG AGG CCA-3′. Oligofectamine (Invitrogen) was used to deliver siRNA (10 nM) into cultured cardiomyocytes according to the manufacturer's protocol. Transfection efficiency was confirmed by Cy3-labeled transfection control and was almost 100% (data not shown). Double-strand scrambled oligonucleotides were used as a negative control. Twenty-four hours after transfection, cells were serum starved for 48 h, and then cell viability was assessed by MTS assay.

Measurement of Cytotoxicity—Cell viability was estimated by MTS assay. Cells were plated on 96-well plates at a density of 1–5 × 10^4 cells/well and were cultured for 2 days. MTS assay was performed by using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) as described in the manufacturer's protocol (10).

Cell damage was evaluated by measuring CPK activity in culture medium by spectrophotometric assay using the CPK test WAKO kit (Wako Chemical) according to the manufacturer's protocol as described previously (27).

Statistical Analysis—Each value was expressed as the means ± S.E. Statistical significance was determined by Student's *t* test. Differences were considered statistically significant when the calculated *p* value was < 0.05.

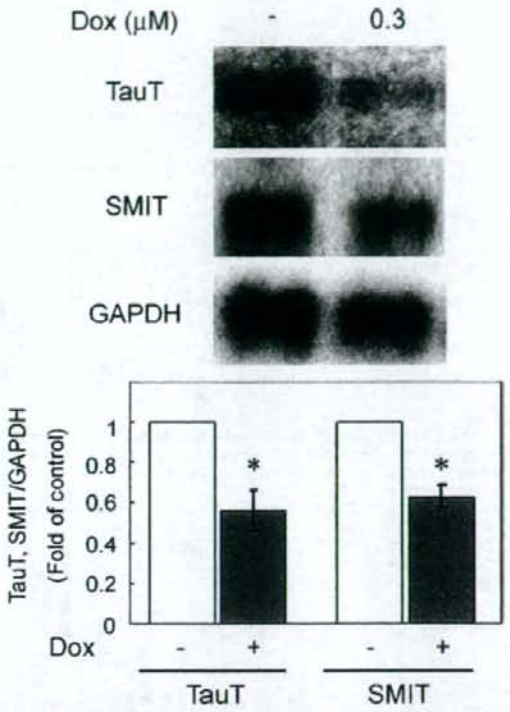

FIGURE 1. **Expression of NFAT5 target genes was down-regulated by Dox exposure in cultured cardiomyocytes.** Northern blot analyses of total RNA extracted from cardiomyocytes cultured with Dox (0.3 μM) for 24 h. Representative bands from three independent cell preparations are shown. Data are mean ± S.E., $n = 3$. *, $p < 0.05$ *versus* Dox (−).

RESULTS

NFAT5 and Its Downstream Targets Are Down-regulated by Dox Exposure in Cultured Cardiomyocytes—Osmotic stress-related genes, such as the organic osmolyte transporters, are expressed in the tissues that are not exposed to hypertonic stresses, including the heart (28). To address their pathophysiological significance in Dox-induced cardiotoxicity, we examined the expression of TauT and SMIT in cardiomyocytes exposed to Dox. Myocytes were treated with or without Dox (0.3 μM) for 24 h, and TauT and SMIT mRNA were analyzed. Northern blot analyses revealed that Dox exposure resulted in down-regulation of TauT and SMIT (Fig. 1).

Because the expression of osmotic stress-related genes is regulated by NFAT5 (7, 8), we tested the effect of Dox on NFAT5 expression in cultured cardiomyocytes. Western blot analyses revealed that NFAT5 protein was significantly reduced compared with non-treated myocytes (Fig. 2, *A* and *D*). Glyceraldehyde-3-phosphate dehydrogenase protein expression was not affected by Dox treatment, suggesting that the reduction of NFAT5 may be due to a selective pathway induced by Dox. NFAT5 was decreased in a time-dependent manner, and the reduction in NFAT5 was examined 6 h after Dox exposure (Fig.

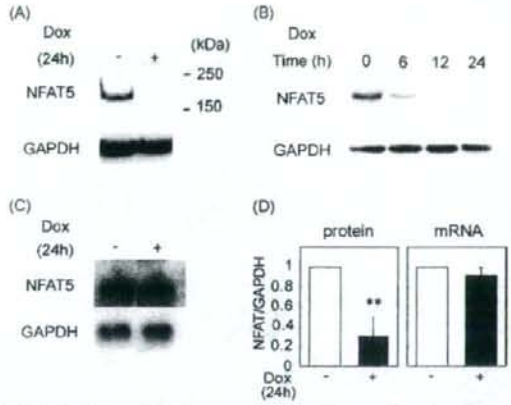

FIGURE 2. **NFAT5 protein, but not mRNA, is decreased by Dox exposure in cardiac myocytes.** *A* and *B*, Western blot analyses of cell lysates prepared from cardiomyocytes exposed to Dox (0.3 μM) for 24 h (*A*) or the indicated timeS (*B*) by using anti-NFAT5 antibody and anti-glyceraldehyde-3-phosphate dehydrogenase antibody. *C*, Northern blot analyses of total RNA prepared from cardiomyocytes exposed to Dox (0.3 μM) for 24 h. *D*, quantification of NFAT5 protein (*left*) and mRNA (*right*) level in cardiomyocytes exposed to Dox for 24 h. Data are mean ± S.E., $n = 5$ (*left*) or 3 (*right*). **, $p < 0.01$ *versus* Dox (−).

2*B*). Together, these findings identified a novel nuclear pathway targeted by Dox in cardiomyocytes.

To ascertain whether a decrease of NFAT5 protein by Dox exposure is dependent on NFAT5 transcript level, Northern blot analyses were performed. NFAT5 mRNA was not down-regulated by Dox exposure (Fig. 2, *C* and *D*). Thus, Dox-mediated decline of NFAT5 protein may result from reduced translation or selective degradation.

Degradation of NFAT5 Is Enhanced by Dox Exposure in Cardiomyocytes—To determine whether NFAT5 stability is affected by Dox exposure, the protein level of NFAT5 was measured in the presence of cycloheximide, a protein synthesis inhibitor, by Western blot analyses (Fig. 3*A*). Dox treatment accelerated turnover of NFAT5 protein in cells treated with cycloheximide. Collectively, these observations indicate that Dox enhances specific proteolysis of NFAT5.

NFAT5 Is Degraded via Proteasome-mediated Proteolysis Pathway—Previous study has demonstrated that Dox activates proteasome-mediated proteolysis pathway in cardiac myocytes (6). To investigate the pathway for reduction of NFAT5, we analyzed whether the proteasome-mediated proteolysis is involved in the reduction of NFAT5 protein by Dox. Cardiom-

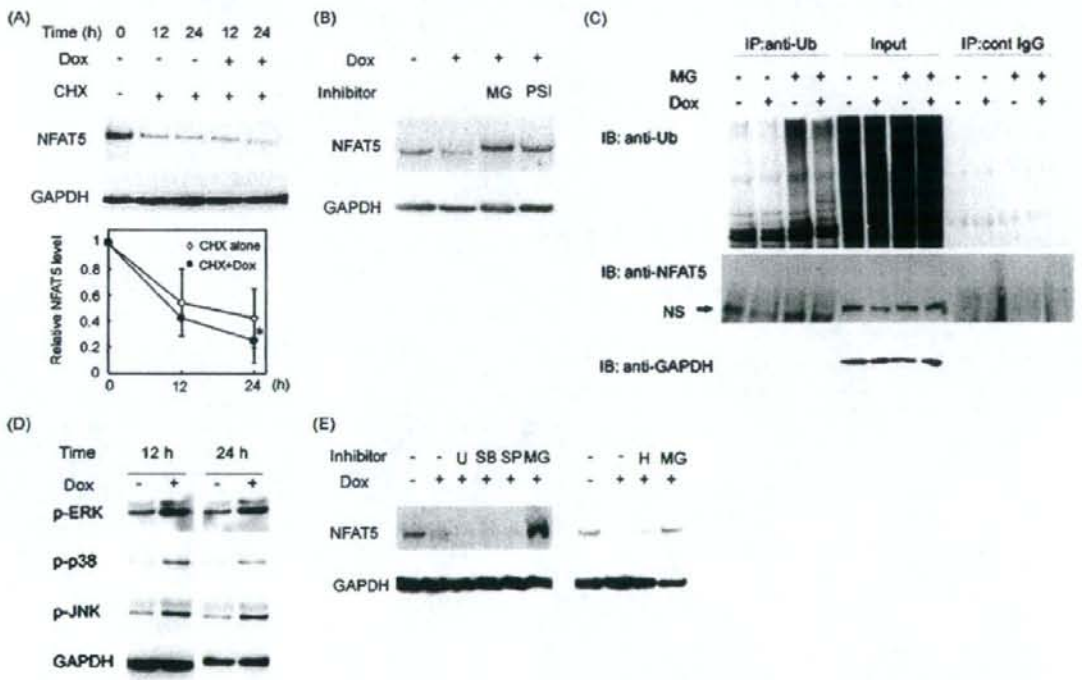

FIGURE 3. **Degradation of NFAT5 protein is increased by Dox exposure and is prevented by treatment with proteasome inhibitors.** *A* and *B*, Western blot analyses of whole cell lysates from cardiomyocytes cultured with inhibitors and/or Dox. *A*, cardiomyocytes were pretreated with cycloheximide (*CHX*) (5 μM) for 1 h and were then exposed to Dox (0.3 μM) for the indicated times. Cycloheximide was often in culture medium. Relative amounts of NFAT5 protein were determined. Data are mean ± S.E., $n = 3$. *, $p < 0.05$ *versus* cycloheximide alone. *B*, cardiomyocytes were pretreated with Me_2SO or proteasome inhibitor MG-132 (*MG*) (5 μM) or proteasome-specific inhibitor I (*PSI*) (10 nM) for 1 h and were then exposed to Dox (0.3 μM) for 24 h. *C*, immunoprecipitation assay of cell lysates from cardiomyocytes exposed to MG-132 and/or Dox for 12 h. Ubiquitinated proteins were precipitated by using anti-ubiquitin antibody and then immunoblotted by anti-ubiquitin and anti-NFAT5 antibodies. *D*, Western blot analyses of whole cell lysates from cardiomyocytes treated with Dox for 12–24 h. Cell lysates were immunoblotted by phosphor-specific antibodies against ERK, MAPK, and JNK. *E*, Western blot analyses of whole cell lysates from cardiomyocytes pretreated with U0126 (*U*) (10 μM), SB2028 (*SB*) (10 μM), SP600125 (*SP*) (10 μM), H-7 (30 μM), and MG-132 (*MG*) (5 μM) for 1 h and exposed to Dox for an additional 24 h. The level of NFAT5 protein was determined. Similar results were obtained from at least three independent cell preparations in these experiments.

yocytes were cultured with Dox in the presence or absence of proteasome inhibitor MG-132 (5 μM) or proteasome-specific inhibitor I (10 nM) for 24 h, and then the expression of NFAT5 was analyzed by Western blot (Fig. 3*B*). While NFAT5 expression was decreased by Dox alone, treatment with proteasome inhibitors prevented the down-regulation of NFAT5. These data suggest that degradation of NFAT5 caused by Dox exposure is due to the activation of proteasome.

To ascertain whether ubiquitination was involved in Dox-mediated NFAT5 degradation, immunoprecipitation assay was performed (Fig. 3*C*). Samples used in this assay were prepared from cultured cardiomyocytes treated with or without Dox and/or MG-132 for 12 h. Whereas ubiquitinated proteins were detected in MG-132- or MG-132/Dox-treated cells, ubiquitinated NFAT5 was not detected in immunoprecipitated proteins with anti-ubiquitin antibody. These data suggest that ubiquitin-independent proteasome proteolysis pathway may be involved in Dox-enhanced NFAT5 degradation.

Various kinds of serine/threonine protein kinases, such as mitogen-activated protein kinase (MAPK) family proteins, are activated by Dox exposure in hearts and cardiomyocytes (29–31). Indeed, we ascertained that extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, and c-Jun N-terminal kinase (JNK) were activated by Dox exposure over 12 h (Fig. 3*D*). We tested whether Dox-induced activation of MAPK families was involved in NFAT5 degradation by Dox exposure using U0126, a MEK 1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase I) inhibitor, or SB203580, a p38 MAPK inhibitor, or SP600125, a JNK inhibitor (Fig. 3*E*). However, none of these inhibitors suppressed Dox-enhanced degradation of NFAT5 protein. Further, we examined the effect of H-7, a potent inhibitor of serine/threonine kinases with relative specificity for protein kinase C. However, H-7 also failed to suppress the NFAT5 degradation. Thus, these data indicate that the signal cascades associated with MAPK family members and H-7-sensitive protein kinases are not involved in Dox-enhanced NFAT5 degradation.

Degradation of NFAT5 Protein Caused by Dox Exposure Is Not Observed in Non-cardiac Cells—To evaluate whether NFAT5 degradation by Dox is a cardiac myocyte-specific response, Western blot was performed by using lysate obtained from cardiac fibroblasts or the HEK293 cell line. Reduction of NFAT5 protein by Dox was not observed in the cell lysates from either cardiac fibroblasts or HEK293 cells, as shown in Fig. 4.

Transcriptional Activity of NFAT5 Is Suppressed by Dox Exposure in Cardiac Myocytes—Next, we determined intracellular localization of NFAT5 in cardiomyocytes exposed to Dox. Immunofluorescence microscopic analyses by using anti-NFAT5 antibody revealed that NFAT5 was localized in nuclei in cardiomyocytes cultured in the absence of Dox, whereas nuclear NFAT5 protein was markedly decreased by exposure to Dox (Fig. 5*A*). Consistently, Western blot analyses showed that NFAT5 was decreased in nuclear extracts from Dox-exposed cardiomyocytes compared with that of control cells (Fig. 5*B*).

To evaluate whether the down-regulation of NFAT5 protein is accompanied by the reduction in its transcriptional activity, promoter-reporter assay was performed by using reporter plasmids consisting of the 5′-flanking region of *TauT* gene (Fig. 6*A*), because the consensus binding sequence for NFAT5 was previously identified at −110 of *TauT* gene (10). Luciferase assays demonstrated that DOX exposure reduced the promoter activity of pTauT/-862-Luc in cardiac myocytes. Dox-dependent down-regulation of promoter activity was also observed in myocytes transfected with pTauT/-124-Luc. The reporter plasmid pTauT/-99-Luc, in which the NFAT5 consensus sequence was deleted, did not show down-regulation of its activity in response to Dox. The mutation of the NFAT5 binding sequence in the pTauT/-124-Luc construct (pTauT/-124mut-Luc) also

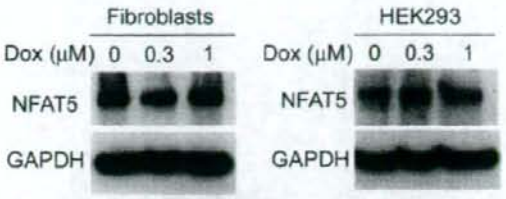

FIGURE 4. **Expression of NFAT5 protein is not influenced by Dox in cardiac fibroblasts and HEK293 cells.** Western blot analyses of cell lysates prepared from cardiac fibroblasts (*left*) and HEK293 cells (*right*) exposed to Dox (0.3–1 μM) for 24 h. Similar results were obtained from three independent cell preparations.

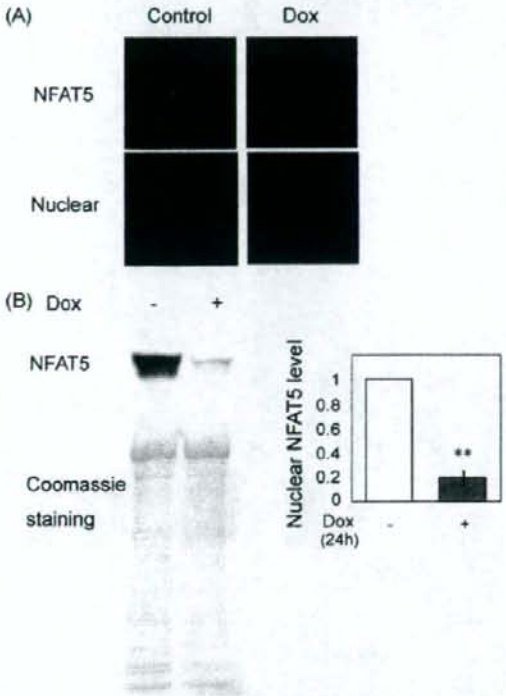

FIGURE 5. **Nuclear NFAT5 protein is reduced by Dox exposure.** *A*, cardiomyocytes were exposed to Dox (0.3 μM) for 24 h and were then assessed by immunocytochemistry using anti-NFAT5 antibody (*green*; *upper panel*), and nuclei were stained by Hoechst33258 (*blue*; *lower panel*). Similar results were obtained from three independent cell preparations. *B*, Western blot analyses of nuclear protein (20 μg) extracted from cardiomyocytes cultured with or without Dox (0.3 μM) for 24 h. Representative bands from three independent cell preparations are shown. The proteins remaining in the gel after transfer were stained with Coomassie brilliant blue to show an equal amount of loading. Data are mean ± S.E., $n = 3$, **, $p < 0.01$ *versus* Dox (−).

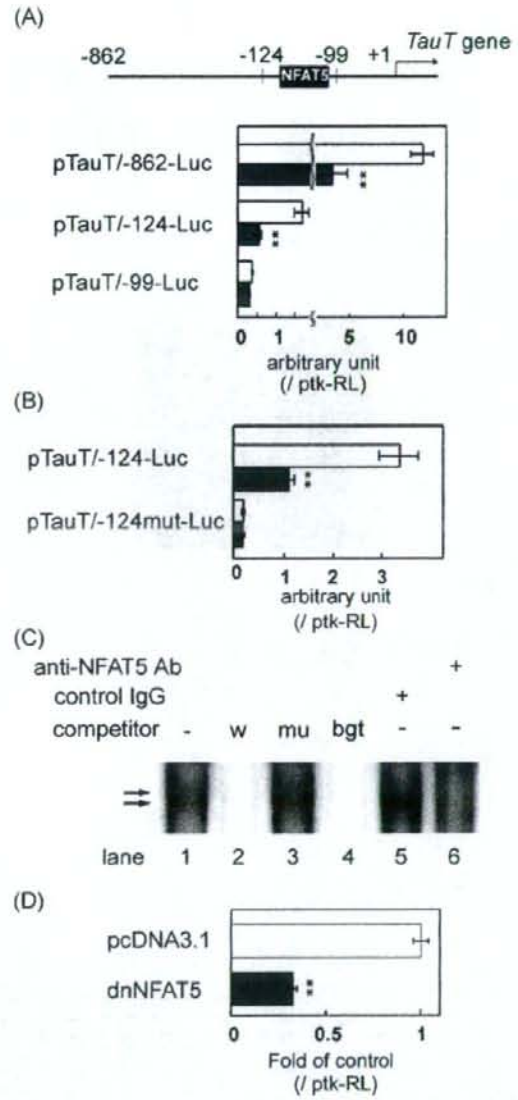

FIGURE 6. **Transcript activity of NFAT5 is suppressed by Dox exposure in cardiomyocytes.** *A* and *B*, cells transfected with promoter-reporter constructs (pTauT/-862-Luc, pTauT/-124-Luc, pTauT/-99-Luc, or pTauT/-124mut-Luc) were exposed to Dox (0.3 μM) for 24 h. Promoter activity was normalized with luciferase activity of pTK-RL. *White* or *gray columns* indicate Dox (−) or (+), respectively. Data are mean ± S.E., $n = 3$. **, $p < 0.01$ versus Dox (−). Each experiment was repeated at least twice with independent cell preparations. *C*, EMSA was performed with nuclear extract from HEK293 cells transfected with pFLAG-NFAT5 and ^{32}P-labeled TauT-NFAT5 oligonucleotide. *W*, wild-type oligonucleotide; *mu*, mutant-type oligonucleotide; *bgt*, NFAT5 binding motif encoded in the 5′-flanking region of the BGT-1 gene (11). *Arrows* indicate NFAT5-DNA complex. Similar results were obtained from three independent experiments. *D*, cells were co-transfected with promoter constructs (pTauT/-124-Luc or pTauT/-124mut-Luc) and expression vector carrying the dominant-negative NFAT5 construct. Empty vector (*pcDNA*) was used as a control of expression vector. Promoter activity was normalized with that of ptk-RL. Data are mean ± S.E., $n = 3$. **, $p < 0.01$ versus pcDNA. Each experiment was repeated three times with independent cell preparations.

remarkably reduced the basal promoter activity, which was not affected by Dox exposure (Fig. 6*B*). To confirm that the NFAT5 binding consensus sequence located in the promoter region of the *TauT* gene actually interacts with NFAT5 protein, EMSA was performed using nuclear extract from HEK293 cells transfected with NFAT5 expression vector (Fig. 6*C*). Protein-DNA complexes were competed by unlabeled probe (*lane 2*) or unlabeled BGT-NFAT5 oligonucleotides that correspond to the NFAT5 consensus sequence in the *BGT-1* gene (11) (*lane 4*) but not by unlabeled oligonucleotides with the consensus sequence mutated (*lane 3*). Further, DNA-protein complex formation was inhibited by anti-NFAT5 antibody (*lane 6*) but not control IgG (*lane 5*), implying that this DNA-protein complex consists of NFAT5.

Consistent with the decrease in promoter activity of *TauT* gene by Dox exposure, the overexpression of dominant-negative NFAT5, which consists of the DNA-binding domain of NFAT5, led to a decrease in promoter activity derived on pTauT/-124-Luc (Fig. 6*D*). Collectively, these data illustrate that degradation of NFAT5 is associated with the down-regulation of the *TauT* gene induced by Dox exposure in cardiomyocytes.

NFAT5 Is Necessary for Myocyte Survival—To determine whether suppression of transcriptional activity of NFAT5 by Dox exposure causes cell toxicity, we tested the effect of NFAT5 inhibition against cardiomyocyte viability. First, we generated adenovirus expressing dnNFAT5 (ad-dnNFAT5) as described under "Experimental Procedures." Cardiomyocytes transfected with ad-dnNFAT5 at multiplicity of infection of 20 exhibited the decreased NFAT5 promoter activity, compared with the cells overexpressing β-galactosidase (data not shown). Ad-dnNFAT5 infection significantly reduced mRNA expression of NFAT5 targets, such as TauT and SMIT, in cardiomyocytes (Fig. 7*D*). After treatment with adenovirus for 48 h, cells were cultured for an additional 24 h in serum-free medium. MTS assay revealed that cell viability was impaired by dnNFAT5 expression (Fig. 7*E*). To quantify the cytotoxic effects of dnNFAT5, CPK leakage into culture medium was measured (Fig. 7*F*). Overexpression of dnNFAT5 resulted in a remarkable increase in CPK leakage into culture medium even in the absence of Dox. Further, we examined the effect of Dox exposure on cell viability in Ad-dnNFAT5-treated cardiomyocytes. CPK leakage level was not equivalent between Dox-treated and -untreated dnNFAT5-overexpressing cells. These data illustrated that NFAT5 is necessary to myocyte survival, and so degradation of NFAT5 by Dox associates Dox-induced cell injury but is not sufficient to lead to Dox-induced cell death.

Next, NFAT5 expression was knocked down by using siRNA specific for its gene. Treatment with siRNA for NFAT5 reduced NFAT5 expression by 80% (Fig. 8*A*). Importantly, myocyte viability was reduced by siRNA targeting NFAT5 gene in a serum-starved condition, compared with scrambled oligonucleotide, as a negative control (Fig. 8*B*). Taken together, our results indicate that NFAT5 is critical for cell survival in cardiomyocytes and that the inactivation of NFAT5 may be involved with Dox-induced cell injury in cardiac myocytes.

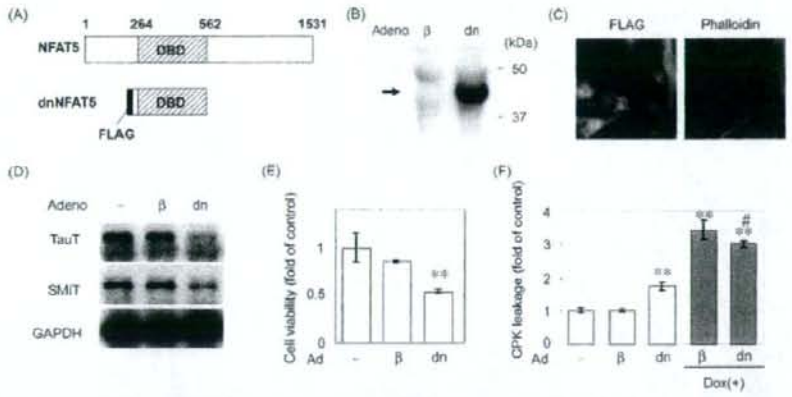

FIGURE 7. **Inhibition of NFAT5 by dominant-negative NFAT5 leads to cell injury in cardiomyocytes.** *A*, construct of the dominant-negative form of NFAT5 (*dnNFAT5*), which consists of isolated DNA-binding domain. *B*, Western blot analyses of cell lysates prepared from cardiomyocytes infected with adenovirus carrying dnNFAT5 (*dn*) or β-galactosidase (*β*) (multiplicity of infection of 20). An *arrow* indicates dnNFAT5. *C*, immunofluorescent microscopic examination of cardiomyocytes infected with adenovirus carrying dnNFAT5 by using anti-FLAG antibody (*green*) or Phalloidin (*red*) to confirm the infection efficiency. *D*, Northern blot analyses of RNA prepared from cardiomyocytes overexpressing dnNFAT5 (*dn*) or β-galactosidase (*β*). *E* and *F*, cell viability by MTS assay (*E*) or measurement of CPK activity of culture medium (*F*) after dnNFAT5 (*dn*)- or β-galactosidase-expressing cardiomyocytes cultured in serum-free medium. Data are mean ± S.E., $n = 4$ (*E*) or 4 (*F*). **, $p < 0.01$ versus β-galactosidase/Dox(−), #, $p < 0.05$ versus dn/Dox(−). Each experiment was repeated at least three times with independent cell preparations.

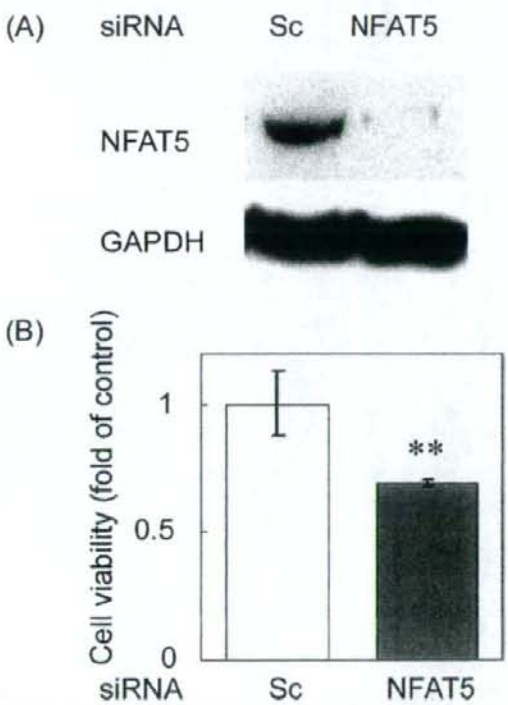

FIGURE 8. **Knock down of NFAT5 gene by siRNA impaired cell viability in cultured cardiomyocytes.** *A*, Western blot analyses of cell lysates from cardiomyocytes treated with 10 nM siRNA targeting the NFAT5 gene (*NFAT5*) or scrambled siRNA (*sc*) as a negative control. *B*, cell viability of cardiomyocytes transfected with siRNA was estimated by MTS assay. After transfection, cells were cultured for an additional 48 h. Data are mean ± S.E., $n = 5$. **, $p < 0.01$ versus scrambled siRNA. The experiment was repeated at least three times with independent cell preparations.

DISCUSSION

In the present study, we demonstrated that the transcriptional activity of NFAT5 was decreased by Dox in cardiac myocytes. The exposure to Dox resulted in the down-regulation of NFAT5 protein in a proteasome-dependent manner in cultured cardiomyocytes, but not in cardiac fibroblasts or kidney cells. Inhibition of NFAT5 activity by either dnNFAT5 or siRNA targeting for NFAT5 resulted in the impairment of cardiomyocyte viability. Collectively, it is suggested that the degradation of NFAT5 protein is a critical event for cardiac-specific cytotoxicity induced by Dox.

It is well known that NFAT5 activity is regulated by extracellular osmolality. Hyperosmotic stress enhances translocation of NFAT5 into the nucleus through phosphorylation by signal molecules, such as ATM, Fyn, p38, and protein kinase A (32–34). In this study, we demonstrated, for the first time, that NFAT5 activity was regulated through the proteasome-mediated proteolysis pathway under cytotoxic conditions, proposing a novel regulatory mechanism of NFAT5 activity. Considering the specificity of the proteasome (35), it is likely that NFAT5 is selectively targeted by proteasome-mediated proteolysis.

We could not detect the ubiquitinated NFAT5 in cardiac myocytes exposed to DOX. In addition to ubiquitin-dependent proteolysis, accumulating evidence demonstrates that proteasome degrades a wide range of proteins, such as ornithine decarboxylase, p53, $p21^{Cip}$, and steroid receptor coactivator-3 (5, 36–40), without the ubiquitinization process. Recently, p300 has been reported to be degraded by Dox exposure via ubiquitin-independent and proteasome-dependent pathway in cultured cardiomyocytes (5), indicating that Dox activates ubiquitin-independent proteolysis through proteasome pathway. Taken together, it is likely that Dox-induced degradation of NFAT5 is mediated by a proteasome-mediated proteolytic process in an ubiquitin-independent manner.

NFAT5 activity is regulated by some protein kinases (32–34). NFAT5 is phosphorylated under hypertonic condition, and NFAT5 phosphorylation is shown to be a critical event for nuclear translocation, followed by DNA binding (41, 42). However, the role of phosphorylation in NFAT5 turnover remains to be addressed. In the present study, we investigated whether phosphorylation signal pathway was associated with Dox-induced NFAT5 degradation by using the specific inhibitors of MAPK family proteins, ERK, p38, and JNK. We also investigated the effects of H7-sensitive protein kinases that regulate cellular events induced by Dox exposure in cardiac myo-

cytes (29, 43). However, we could not find any evidence for the causality between NFAT5 phosphorylation and degradation.

Interestingly, the down-regulation of NFAT5 was observed in cardiac myocytes, but not in cardiac fibroblasts or in kidney cells. A cardiac-specific system may be involved in the degradation of NFAT5. This finding may provide a hint toward future study.

It is noteworthy that NFAT5 activity is regulated by Dox differently from the other NFAT family proteins. In contrast to NFAT5, the other rel/NFκB/NFAT family proteins, such as NFAT4 and NFκB, are activated by Dox through Ca^{2+}/calcineurin and Fas/Fas ligand pathways in cardiac myocytes, respectively (44, 45). Because NFAT5 lacks a calcineurin-regulatory site (8), degradation of NFAT5 is likely to be independent of the calcineurin system. Interestingly, activation of NFAT4 and NFκB has been shown to mediate apoptotic cell death in response to Dox exposure in cardiomyocytes (44, 45), whereas the inactivation of NFAT5 resulted in an increase in susceptibility to cell damage as shown in this study. Thus, Dox may dually mediate cell death signals, partially by inactivating NFAT5 through proteolysis and partially by activating the other NFAT proteins through the Ca^{2+}/calcineurin systems.

Several data presented here illustrate the physiological significance of NFAT5 for cell survival in cardiac myocytes, consistent with previous reports concerning the cytoprotective roles of the transcriptional targets of NFAT5 in mammalian cells (16, 19, 20). TauT is a major factor in maintaining a high gradient of cardiac tissue/plasma taurine concentration (46, 47). The taurine/TauT system protects cardiac tissues against Dox-induced cardiotoxicity, hypoxia-induced apoptosis, and heart failure (48–52). Similarly, *myo*-inositol and betaine, organic osmolytes, play protective roles against various stimulations as well as hyperosmotic stress through their transporter systems, SMIT and BGT-1, respectively, in several types of cells and organs (53, 54), although further studies will be required to elucidate their biological functions in cardiac myocytes. Moreover, Hsp70-2 preserves myocardial function and prevents apoptosis (55, 56). Importantly, Hsp70-2 has been demonstrated to be involved in the cytoprotective process against Dox exposure (57). Collectively, it is suggested that the combined effects of these downstream targets contribute to the cytoprotective functions of NFAT5.

In summary, the present study demonstrates the novel mechanism of Dox-induced cardiotoxicity through NFAT5 pathway in cardiomyocytes. Importantly, this study indicates that degradation of NFAT5 may be due to proteasome pathway activated by Dox. Proteasome-mediated proteolysis is involved in a wide variety of cardiovascular pathophysiology, such as ischemia or ischemia/reperfusion injury (58). Thus, it could be proposed that the reinforcement of NFAT5 activities may represent a new cardioprotective strategy against cardiovascular diseases.

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