Hyperosmotic stress stimulates autophagy via the NFAT5/mTOR pathway in cardiomyocytes

HONG ZHU[1], WEI CAO[2], PENG ZHAO[3], JIEYU WANG[1], YUYING QIAN[1] and YUN LI[1]

[1]Department of Geriatrics, Xuanwu Hospital, Capital Medical University, Beijing 100053; [2]Department of Cardiology, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150081, P.R. China; [3]Division of Cardiology, Department of Internal Medicine, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, CA 90095, USA

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Abstract. Hyperosmotic stress may be initiated during a diverse range pathological circumstances, which in turn results in tissue damage. In this process, the activation of survival signaling, which has the capacity to restore cell homeostasis, determines cell fate. Autophagy is responsible for cell survival and is activated by various pathological stimuli. However, its interplay with hyperosmotic stress and its effect on terminally differentiated cardiac myocytes is unknown. Nuclear factor of activated T-cells 5 (NFAT5), an osmo-sensitive transcription factor, mediates the expression of cell survival associated-genes under hyperosmotic conditions. The present study investigated whether NFAT5 signaling is required in hyperosmotic stress-induced autophagy. It was demonstrated that the presence of a hyperosmotic stress induced an increase in NFAT5 expression, which in turn triggered autophagy through autophagy-related protein 5 (Atg5) activation. By contrast, NFAT5 silencing inhibited DNA damage response 1 protein expression, which then initiated the activation of mammalian target of rapamycin signaling. Therefore, the balance between NFAT5-induced apoptosis and autophagy may serve a critical role in the determination of the fate of cardiomyocytes under hyperosmotic stress. These data suggest that autophagy activation is a beneficial adaptive response to attenuate hyperosmotic stress-induced cell death. Therefore, increasing autophagy through activation of NFAT5 may provide a novel cardioprotective strategy against hyperosmotic stress-induced damage.

Correspondence to: Dr Yun Li, Department of Geriatrics, Xuanwu Hospital, Capital Medical University, 45 Chang Chun Street, Beijing 100053 P.R. China
E-mail: liy_xw@sina.com

Abbreviations: LC3, microtubule-associated protein 1 light chain 3; NFAT, nuclear factor of activated T-cell; Atg, autophagy-related; SQSTM1, sequestosome-1; PE, phosphatidylethanolamine; REDD1, DNA damage response 1

Key words: hyperosmotic stress, nuclear factor of activated T-cells 5, cardioprotective, autophagy, cardiomyocytes

Introduction

Various pathological conditions, including ischemia, septic shock and diabetic comas, are accompanied by osmotic changes which may subsequently induce cellular death and tissue damage (1). The underlying mechanism may be associated with the activation of survival signaling cascades under hyperosmotic stress, which leads to the disruption of the homeostasis between cell death and survival pathways, and the subsequent accumulation of damaged, aggregated, misfolded and oxidized proteins. It was demonstrated that rapid cardiomyocyte apoptosis was simulated under hyperosmotic stress *in vitro* (2), which was associated with elevated autophagy (3,4). However, the underlying mechanism has not been fully elucidated.

Autophagy is a highly conserved pathway in eukaryotes, serving a critical role in cellular survival during pathophysiological processes and hyperosmotic stress responses (5-8). When autophagy is initiated, autophagosome-lysosome fusion degrades cytosolic cargos encapsulated by double membranous autophagosomes, which are tagged by lipid-conjugated microtubule-associated protein 1 light chain 3 (LC3-II) (9). Therefore, LC3-II is usually regarded as a marker of autophagy (10). In addition, Beclin-1, an additional autophagy marker, serves an essential role in inducing precursor membrane vesicle expansion through the recruitment of additional autophagy-related (Atg) proteins at the onset of the autophagy cascade. The subsequent selective degradation of sequestosome-1 (SQSTM1/p62) represents the accumulation of protein aggregates and the degree of autophagy activation (11). Notably, Atg5 is a critical factor for regulating the maturation of autophagosomes, as Atg5-12 conjugate interacts with Atg16 like 1 to tether the complex to phagophores and autophagosomes. Therefore, Atg5 is considered a key element for autophagy under the majority of circumstances, though Atg5-independent autophagy has been described previously (12).

The nuclear factor of activated T-cells 5 (NFAT5) protein of the NFAT family, has a large C-terminal region

that harbors a hypertonicity-sensitive transactivation domain. Accumulating evidence suggests that NFAT5 is an osmo-sensitive transcription factor, associated with regulating the expression of genes responsible for cell survival under hyperosmotic conditions (13-15). In addition, NFAT5 has been suggested to increase DNA damage response 1 (REDD1) protein and mammalian target of rapamycin (mTOR) complex 1 repressor expression, and inhibit mTOR signaling (16,17); these processes subsequently initiate the autophagic process through autophagy-associated proteins, including BECN1 and Atg5-Atg12. Therefore, it is possible that the NFAT5 pathway affects the autophagic pathway under hyperosmotic stress. However, little is known about the hyperosmotic stress-induced autophagic mechanism in terminally differentiated cardiomyocytes.

The present study investigated the role of hyperosmotic stress-induced autophagy in cardiomyocytes. It was demonstrated that hyperosmotic stress increased NFAT5 expression, which then triggered autophagy through Atg5 activity. These data provided novel insights into the role of NFAT5 in regulating autophagy in cardiomyocytes under hyperosmotic stress.

Materials and methods

Ethics statement. The present study was approved by the Ethics Committee of Xuanwu Hospital Capital Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) (18). All animals were treated in accordance with guidelines established by the Capital Medical University Institutional Animal Care and Use Committee.

Cell culture and treatment. Briefly, 120 male Sprague-Dawley rats (3 months old, ~200 g) were obtained from the Laboratory Animal Center of Capital Medical University (Beijing, China). Rats were individually housed at 22˚C with a relative humidity of 55%, acclimatized for ≥1 week, under a 12 h light/dark cycle prior to experimentation. Water and food were provided *ad libitum*. The rats were anesthetized and heparinized. Subsequent to anaesthetizing the animals with phenobarbital (150 mg/kg body weight), the rats were sacrificed by excision of the heart. The rapidly excised hearts were mounted on a Langendorff perfusion apparatus, and immediately perfused with Ca_2^+-free buffer (120.4 mM NaCl, 14.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$-$7H_2O$, 10 mM Na-HEPES, 4.6 mM $NaHCO_3$, 30 mM taurine, 10 mM 2,3-butanedione monoxime and 5.5 mM glucose, pH 7.1). Enzymatic digestion was followed by adding 1.5 mg/ml collagenase II (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and $CaCl_2$ (50 mM) to the perfusion solution. Following digestion for 20 min, the ventricles were quickly removed and digested in the perfusion solution, with 10% calf serum (Chemicon International Inc., Temecula, CA, USA). and 12.5 mM $CaCl_2$. The solution was then filtered with 40 mm cell strainer (Thermo Fisher Scientific, Inc.) to exclude undigested tissues. Following centrifugation of the cardiomyocytes at 150 x g at 4˚C for 10 min, the supernatant was aspirated, and the cardiomyocytes were resuspended with M199 medium with selenium-insulin-transferrin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 6-well culture plates. The final cell suspension, containing 70-80% viable myocytes, was used in subsequent experiments.

Conditioned medium was prepared as follows: Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA; 270 mOsm/l, control medium) was modified to reach 300, 400, 500 and 600 mOsm by adding NaCl to the medium for 12 h at 37˚C, which was confirmed with a Vapro vapor pressure osmometer (model 5500, Wescor, Inc., Logan, UT, USA). The time-dependent effects of osmotic stress on autophagy were evaluated at various time point. Chloroquine (CQ; 5 μM) was used to treat cells 1 h prior to osmotic stimulation in order to detect autophagy flux.

Gene silencing with small interfering RNA (siRNA). The sequence for siRNAs were as following: Scrambled siRNA, 5'-CUACGCUGAGUACUUCGATT-3'; Beclin-1 siRNA, 5'-CTCAGGAGAGGAGCCATTT-3'; Atg5 siRNA, 5'-GGC CUUUCAUUCAGAAGCUTT-3'; NFAT5 siRNA, 5'-CCA GTTCCTACAATGATAA-3'. All siRNA (200 nM) and transfection reagent (Lipofectamine® 3000; Thermo Fisher Scientific, Inc.) were obtained from Applied Biosystems; Thermo Fisher Scientific, Inc. Transfection with siRNAs was conducted according to the manufacturer's protocol. After 48 h, the efficiency of siRNA-silenced genes was confirmed by western blot analysis as described subsequently, which was conducted to detect the targeted proteins using specific antibodies.

Western blot analysis. Heart tissue were added into tubes with lysis (cat. no. 1632086, Thermo Fisher Scientific, Inc.) for 1 h at 4˚C and the supernatants were collected following centrifugation (11,000 x g for 10 min at 4˚C). Subsequently, the protein concentration of heart lysates was quantified using bovine serum albumin (cat. no. A2058, Sigma-Aldrich; Merck KGaA) and measured via the absorption of Coomassie Brilliant Blue in the spectrophotometer. Thereafter, the samples were frozen at -20˚C until use. Protein samples were loaded onto SDS page (4-15%) for separation (10 μl per lane). The separated proteins were then transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 1 h in TBS containing 0.1% Tween-20. Incubation with primary antibodies [LC-3 (cat. no. 4108); Beclin-1 (cat. no. 3495); Atg5 (cat. no. 12994); SQSTM1/p62 (cat. no. 39749); ribosomal portion S6 (S6; cat. no. 2317S); phosphorylated p-S6 (cat. no. 4858S); pro-caspase-3 (cat. no. 9664); caspase-3 (cat. no. 9662); and GAPDH (cat. no. 2118); all from Cell Signaling Technology, Inc., Danvers, MA, USA] and [REDD1 (cat. no. ABC245) and NFAT5 (cat. no. SAB2107944); all from Sigma-Aldrich; Merck KGaA] was performed at 4˚C overnight. All antibodies were used at a dilution of 1:1,000. Secondary antibody incubation at 1:2,000 dilution was then performed for 60 min at 37˚C with peroxidase-conjugated Affinipure goat anti-rabbit IgG (H+L, Cell Signaling Technology, Inc.; cat. no. 7074) or anti-mouse IgG (H+L, Cell Signaling Technology, Inc.; cat. no. 7076)-labeled secondary antibodies. Following washing 3 times, the membranes were subjected to enhanced chemiluminescent detection with BeyoECL Plus (Beyotime Institute of Biotechnology, Haimen, China). Subsequent to exposure

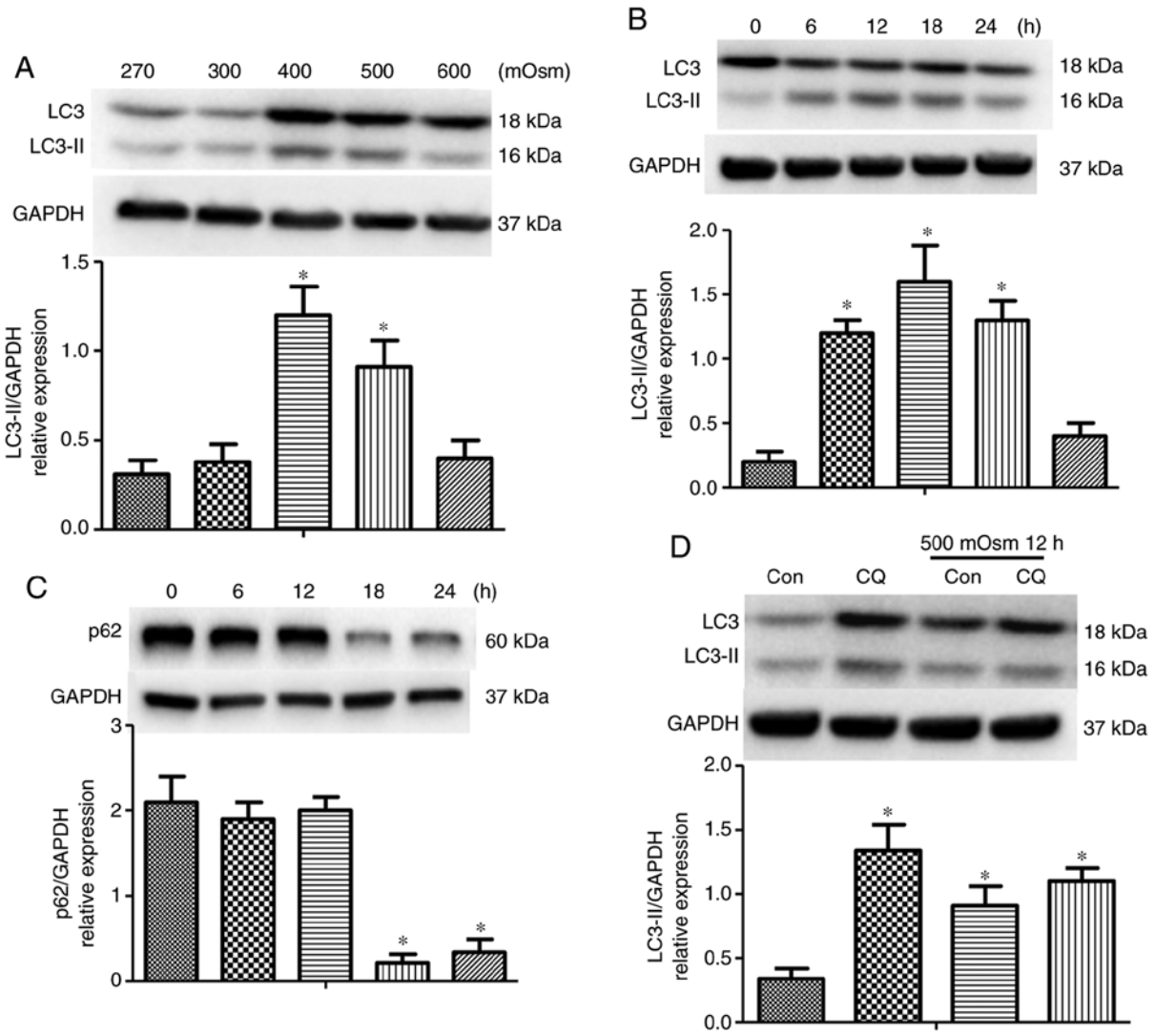

Figure 1. Hyperosmotic stress activates autophagy in cardiomyocytes. (A) Western blot analysis of LC3-II protein levels in cardiomyocytes which were treated with different concentrations of conditioned medium up to 12 h at 37˚C. (B) Western blot analysis of LC3-II protein expression which is regulated by NaCl in a time-dependent manner in cardiomyocytes. Elevation of LC3-II protein expression levels peaked at 12 h, followed by a decrease in LC3-II from 18 to 24 h subsequent to 500 mOsm stimulation. (C) Western blot analysis of sequestosome-1/p62 levels in cardiomyocytes stimulated with 500 mOsm NaCl were significantly reduced from 18 and 24 h following treatment. (D) Western blot analysis of LC3-II protein levels in cardiomyocytes treated with 5 μM lysosomal inhibitor CQ. The graphs next to each blot represent quantification of pooled data from at least three independent experiments. Data are expressed as mean ± standard deviation. *P<0.05 vs. control. LC3, microtubule-associated protein 1 light chain 3; LC3-II, lipid-conjugated LC3; p62, sequestosome-1; Con, control; CQ, chloroquine.

to X-ray film, the membranes were stripped in 5 ml stripping buffer (Bejing CoWin Biotech, Beijing, China) for 15 min at room temperature and re-incubated with an antibody against histone (Cell Signaling Technology, Inc.; cat. no. 7631; 1:1,000) for normalization. Densitometric analysis was performed using the Tanon 3500/3500R Tanon Gel Imaging System (Tanon Science and Technology Co., Ltd., Shanghai, China).

Quantitative detection of cell apoptosis. Apoptosis was analyzed using a fluorescein isothiocyanate-conjugated Annexin V Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. In brief, cells were harvested by trypsin treatment and subsequently labeled with Annexin V and propidium iodide for 15 min at 37˚C. Labeled cells were analyzed with a FACScan flow cytometer (BD Biosciences) and CellQuest software (version 5.1; BD Biosciences).

Statistical analysis. Statistical analysis was performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). All results were presented as mean ± standard deviation. Comparisons of all pairs were performed using a paired Student's t-test. Comparisons of means of multiple groups were performed by one-way analysis of variance followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Hyperosmotic stress activates autophagy in cardiomyocytes. To investigate the effect of hyperosmotic stress on autophagy, cardiomyocytes were treated with different concentrations of NaCl as aforementioned for up to 12 h; the levels of LC3 and LC3-II were then detected. The expression of LC3-II protein began to accumulate at 400 mOsm and peaked at 500 mOsm

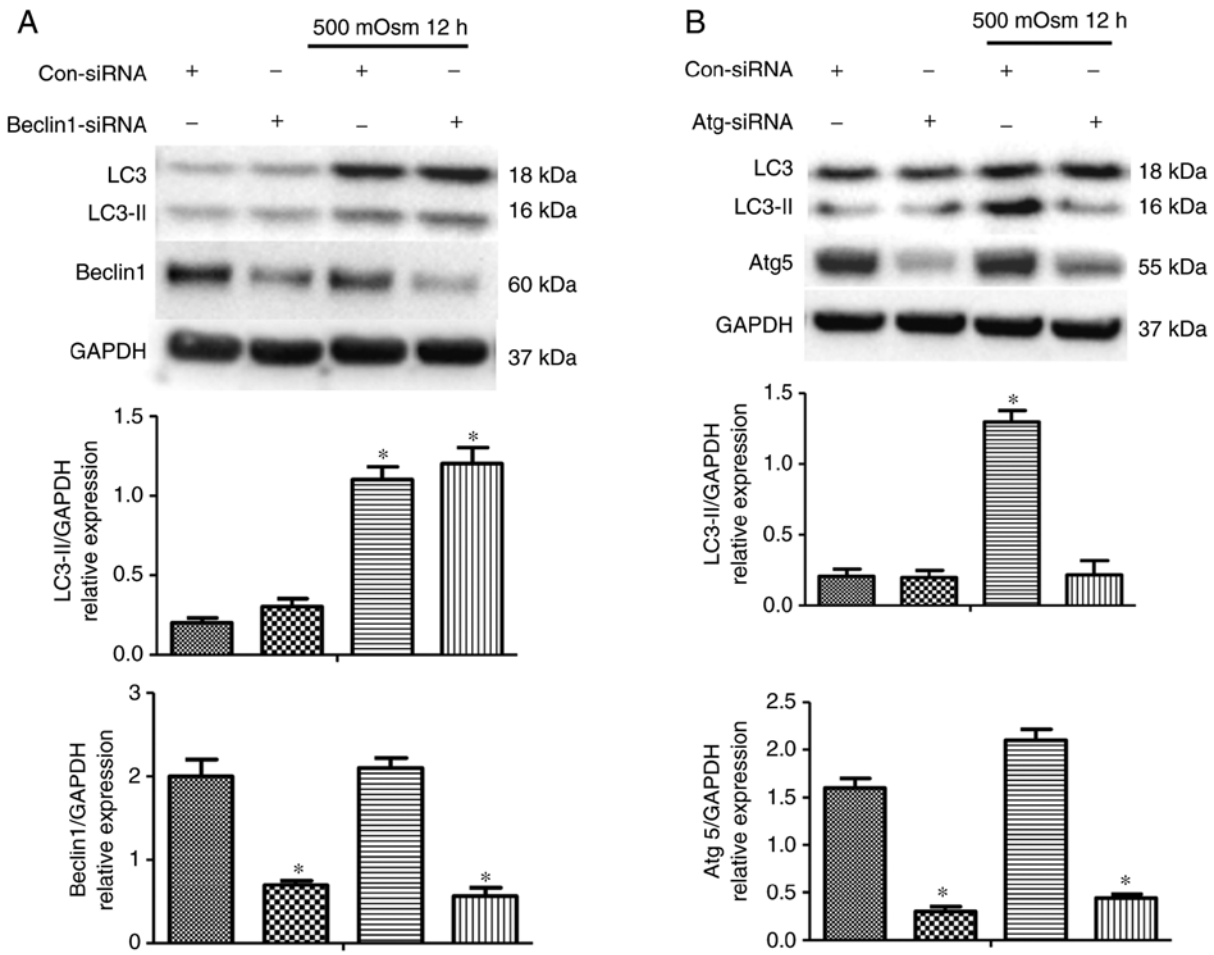

Figure 2. Atg5 is involved in hyperosmotic stress-induced autophagy. Representative images of western blot analyses indicate protein expression levels of (A) LC3-II and Beclin-1, and (B) LC3-II and Atg5. The graphs next to each blot represent quantification of pooled data from at least three independent experiments. Data are expressed as mean ± standard deviation. *P<0.05 vs. control. LC3, microtubule-associated protein 1 light chain 3; LC3-II, lipid-conjugated LC3; Atg5, autophagy related protein 5; siRNA, small interfering RNA; Con, control.

(Fig. 1A). In addition, time-dependent changes in autophagy were determined in cardiomyocytes with 500 mOsm stimulation. Elevation in autophagy reached a peak at 12 h, followed by a decrease in LC3-II from 18 to 24 h subsequent to 500 mOsm stimulation (Fig. 1B). The level of SQSTM1/p62, an autophagosome cargo protein that is concomitantly degraded with increases in levels of autophagy (19), was then measured. The SQSTM1/p62 protein was degraded when cardiomyocytes were stimulated with 500 mOsm from 18 to 24 h, implying a continuous increase in autophagy flux (Fig. 1C). Increases in autophagosome level may be caused either by increased formation of new autophagosomes, or by decreased autophagosome degradation (20). To examine the effect of hyperosmotic stress on autophagic flux, cells were treated with 5 μM lysosomal inhibitor CQ. Increased LC3 II levels, indicated by the western blot analysis results, suggested that treatment with CQ increased hyperosmotic stress-induced autophagy. In addition, CQ application induced additional increases in autophagosome formation in cardiomyocytes (Fig. 1D). Therefore, these results indicated that instead of decreasing its degradation, hyperosmotic stress induced new autophagosome formation in cardiomyocytes.

Atg5 is involved in hyperosmotic stress-induced autophagy. To additionally elucidate the downstream mediators involved in hyperosmotic stress-induced autophagy, the classic downstream components of the autophagy process were inhibited using siRNAs. As Beclin-1 is responsible for the formation of new autophagosomes, its involvement in hyperosmotic stress-induced autophagy was examined through treatment with a Beclin-1-specific siRNA, which significantly decreased Beclin-1 protein level. Contrary to our hypothesis, the decrease in Beclin-1 did not change the level of hyperosmotic stress-induced LC3-II accumulation (Fig. 2A), suggesting that Beclin-1 may not be involved in hyperosmotic stress-induced autophagy. The role of Atg5, a known factor associated with the processes of various stress-induced transcription factors and protein kinases, was then examined. Using an siRNA specifically against Atg5, it was identified that silencing Atg5 attenuated the increase in LC3-II expression compared with Con-SiRNA group when the cells were treated with 500 mOsm medium for 12 h (Fig. 2B). This result indicated that Atg5 was required for hyperosmotic stress-induced autophagy.

Hyperosmotic stress-induced autophagy is regulated by NFAT5 in cardiomyocytes. To examine the hypothesis that the interplay between autophagy and NFAT5 pathways may affect cell survival under hyperosmotic conditions, the role of NFAT5 in hyperosmotic stress-induced autophagy and its

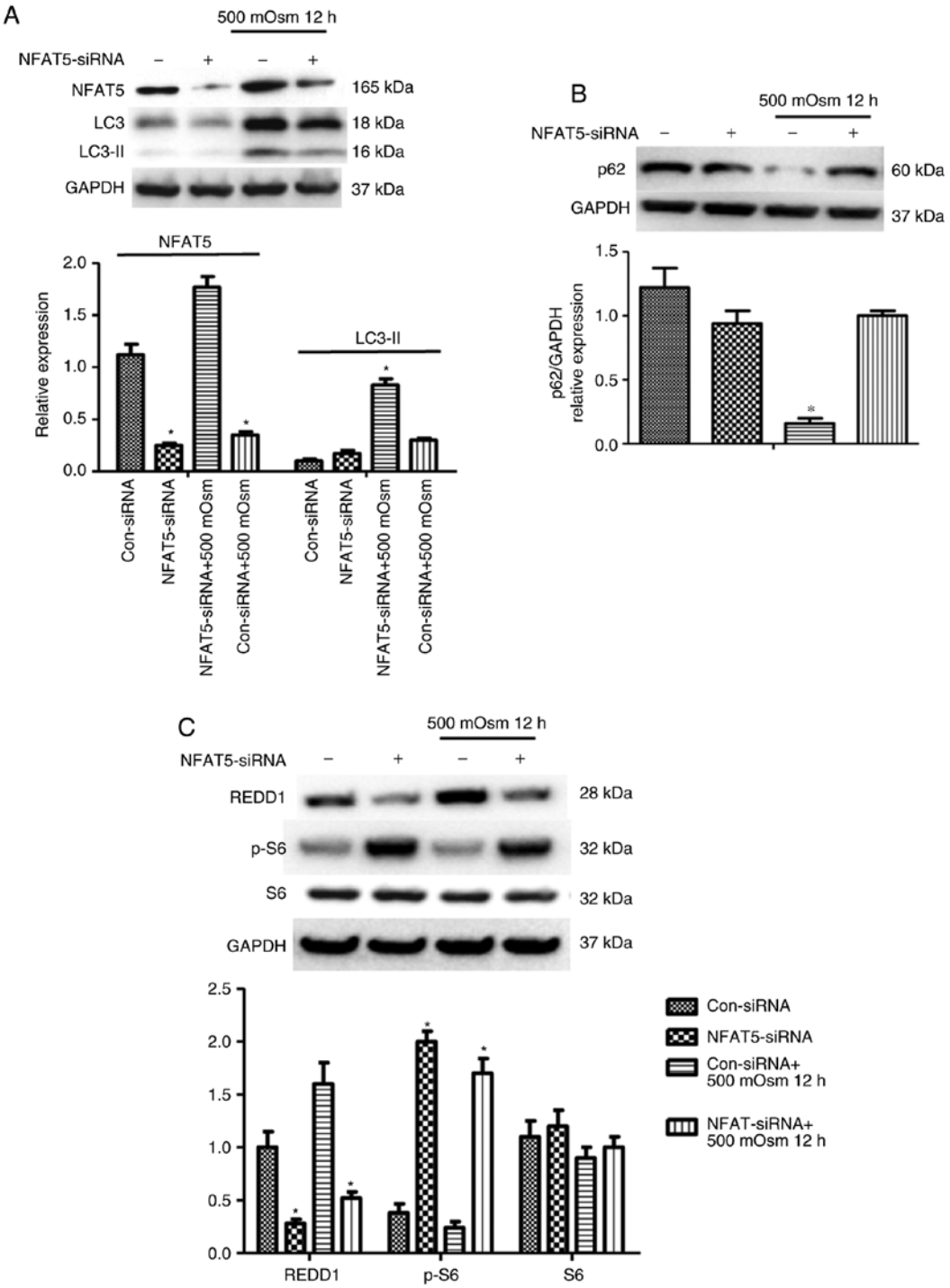

Figure 3. Hyperosmotic stress-induced autophagy is regulated by NFAT5 in cardiomyocytes. Representative images of western blot analyses indicate protein expression levels of (A) NFAT5 and LC3-II; (B) p62; and (C) REDD1, p-S6 and S6 proteins. The graphs next to each blot represent quantification of pooled data from at least three independent experiments. Data are expressed as mean ± standard deviation. *P<0.05 vs. control. NFAT5, nuclear factor of activated T-cells 5; LC3, microtubule-associated protein 1 light chain 3; LC3-II, lipid-conjugated LC3; p62, sequestosome-1; REDD1, DNA damage response l; p, phosphorylated; S6, ribosomal portion S6.

interaction with autophagy-associated proteins was additionally investigated. Cells were treated with 500 mOsm following transfection with an siRNA control or siRNA against NFAT5. The expression of NFAT5 was significantly decreased following siRNA treatment, which was accompanied with decreased hyperosmotic stress-induced LC3-II accumulation as determined by western blot analysis (Fig. 3A). A similar result was observed for SQSTM1/p62 (Fig. 3B); SQSTM1/p62 accumulation was identified in NFAT5 siRNA-treated cells, which may be an essential response to hyperosmotic stress. Furthermore, the absence of NFAT5 prevented REDD1 expression, and caused an increase in the phosphorylation of S6 (Fig. 3C), a downstream mediator of mTOR whose phosphorylation typically decreases during autophagic process. Therefore, these results implied that NFAT5 regulated the hyperosmotic stress-induced mTOR pathway by activating REDD1.

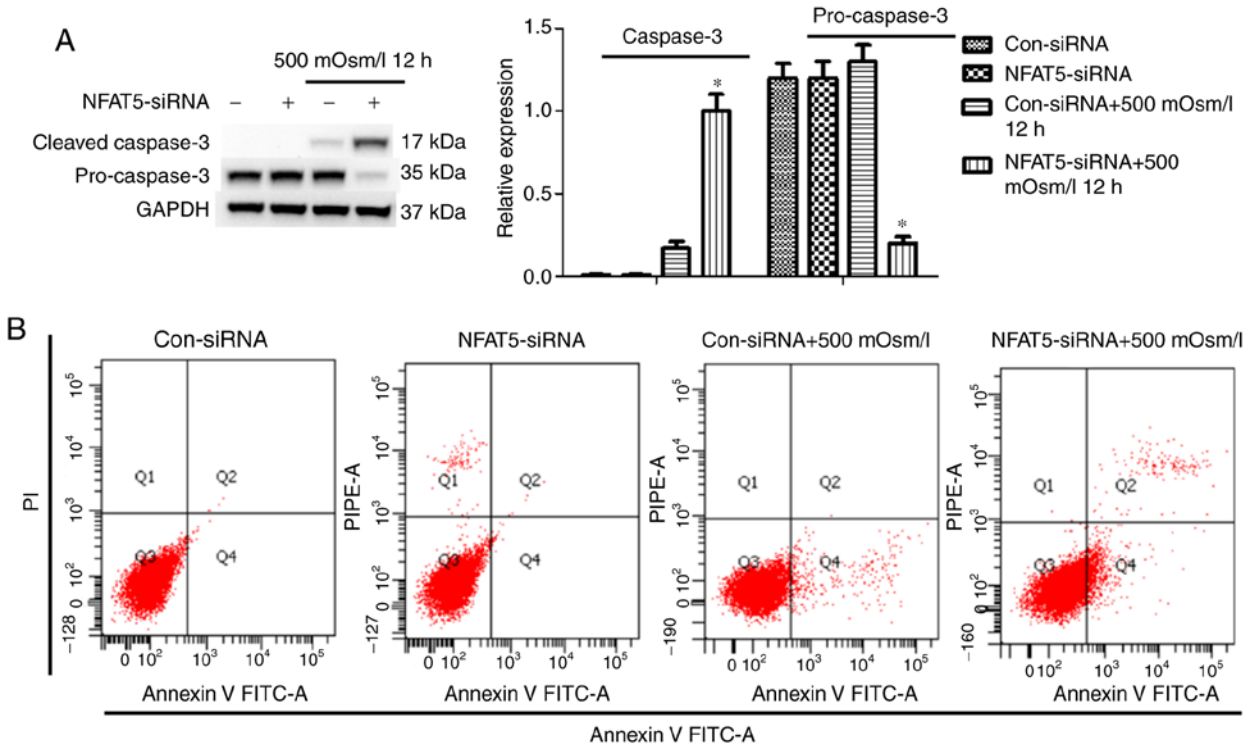

Figure 4. NFAT5 is the key modulator that controls the balance of apoptosis and autophagy in response to hyperosmotic stress. (A) Representative images of western blot analyses indicate protein expression levels of cleaved-caspase 3 and pro-caspase 3. The graphs next to each blot represent quantification of pooled data from at least three independent experiments. Data are presented as mean ± standard deviation. *P<0.05 vs. control. (B) Representative flow cytometric analysis of cardiomyocytes. NFAT5, nuclear factor of activated T-cells 5; Con, control; siRNA, small interfering RNA; PI FITC-A, fluorescein isothiocyanate A.

NFAT5 mediates the balance of apoptosis and autophagy in response to hyperosmotic stress. To determine the role of NFAT5 in regulating autophagy and cell death, cell viability and cell death were examined when treated with, or without, NFAT5 siRNA. Western blot analysis indicated that NFAT5 downregulation exacerbated hyperosmotic stress-induced cell death in cardiomyocytes, with higher levels of cleaved caspase 3 and an increased number of apoptotic cells. (Fig. 4A and B). Overall, these observations supported the hypothesis that attenuated NFAT5 levels aggravated hyperosmotic stress-induced cell death.

Discussion

In the present study, it was revealed that hyperosmotic stress increased NFAT5 expression, which triggered autophagy by the activation of Atg5. Conversely, silencing of NFAT5 by siRNA activated mTOR signaling and decreased REDD1 expression, and resulted in the impairment of cardiomyocyte viability. Collectively, these results suggested that NFAT5 served an essential role in protecting cardiomyocytes from hyperosmotic stress-induced cell death. Various cellular processes may be triggered by dynamic fluctuations in extracellular osmolality (21). The present study used a hyperrosmotic stress condition (500 mOsm/kg) to induce a cellular self-protective response in cardiomyocytes, which may involve diverse compensatory mechanisms, including the accumulation of organic osmolytes and autophagy (21-23). While autophagy serves a protective role against hypertonic stress in certain cell types (25-27), a balance between biosynthetic and catabolic processes, which includes the degradation of entire organelles by autophagy, is required to maintain cell homeostasis.

In general, autophagy is regarded as a means of promoting the cell survival response. However, morphological features of autophagy have also been identified in dying cells. Although it is well known that autophagy is involved in the process of cell death and apoptosis, the role of autophagy in cardiomyocytes under hyperosmotic stress has not been fully elucidated. Previous studies have indicated that hyperosmotic stress induces cardiac apoptosis in a tumor protein p53 (p53)-independent manner, which involves a proportion of translocated p53 into the mitochondria during this process (28,29). The process of p53-mediated cytochrome C release partially involves the disruption of the B-cell lymphoma (Bcl-2)-associated X protein-Bcl-2 and/or the poly [adenosine 5'-diphosphate-ribose] polymerase 9-myeloid leukemia cell-1 complexes (30,31). Nevertheless, previous studies clearly demonstrated that autophagic cell death is regulated by a molecular mechanism distinct from that of apoptosis, though Beclin-1 and Bcl-2 may cooperate with Atg5 to regulate autophagy and apoptosis (32). The effectiveness of Atg5-induced cell death may be overidden by a higher level of Bcl-2, supporting the hypothesis that truncated Atg5 targets mitochondria to release cytochrome C, and perhaps other pro-apoptotic factors (33). Therefore, Atg5 may serve a critical role as a checkpoint responsible for switching from apoptosis to autophagic cell death.

It is well demonstrated that the NFAT5 activity is regulated by extracellular osmolality. Hyperosmotic stress, through the phosphorylation of signal molecules, including Fyn (34), p38

mitogen-activated protein kinase (p38 MAPK) and protein kinase A (35-37), increases the NFAT5 translocation into nuclei. NFAT5, as a ubiquitously expressed transcription factor, regulates the expression of context-dependent gene products in a number of different cell types. Additionally, NFAT5 serves a role in regulating cell migration and proliferation-associated gene expression (38-40). Despite the involvement of multiple kinases in controlling of NFAT5 activity (38,41), neither p38 MAPK nor extracellular signal-regulated kinase 1/2-dependent prototypic stretch-activated kinase pathways are involved in promoting NFAT5 translocation into the nuclei.

P38 MAPK is a key regulator in directing cellular apoptosis, cell cycle arrest, growth inhibition and differentiation (42). P38 MAPK serves positive and negative roles to regulate autophagy: A previous study has indicated that MAPK14/p38a, through activating survival autophagy, confers irinotecan resistance to p53-deficient cells (43). Conversely, it has been demonstrated that suppression of the p38 signaling cascade in tumor necrosis factor-α-treated L929 cells promotes necroptotic and autophagic cell death (44). Although the molecular mechanisms between p38 regulation and autophagy remain largely unknown, certain molecular targets have been suggested in the up- or downregulation of autophagy. The phosphorylation of Atg5 at threonine 75 through p38 MAPK may inhibit starvation-induced autophagy (45). Therefore, the data concerning p38 involvement in the control of the autophagy-apoptosis balance may provide a novel direction for future study.

In summary, the results of the present study demonstrated that hyperosmotic stress enhanced NFAT5 translocation, which in turn stimulated autophagy through Atg5 activity. Conversely, the absence of NFAT5 prevented REDD1 expression and upregulated hyperosmotic stress-induced apoptosis. These data suggested that autophagy activation is a beneficial adaptive response to attenuate hyperosmotic stress-induced cell death. Therefore, increasing NFAT5 activity may represent a novel therapeutic strategy for the protection of cardiomyocytes against hyperosmotic stress-induced damage.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HZ and WC performed the majority of the experiments. HZ and YL made substantial contributions to the design of the present study. PZ, JW completed statistical analysis and data presentation. YQ and HZ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Xuanwu Hospital Capital Medical University, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (18). All animals were treated in accordance with guidelines established by the Capital Medical University Institutional Animal Care and Use Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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