Heavy metal scavenger metallothionein mitigates deep hypothermia-induced myocardial contractile anomalies: role of autophagy

Shasha Jiang,1,2* Rui Guo,2* Yingmei Zhang,2,3 Yunzeng Zou,1 and Jun Ren1,2,3

1Institutes of Biomedical Sciences, Fudan University, Shanghai, China; 2Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, Laramie, Wyoming; and 3Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi’an, China

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Jiang S, Guo R, Zhang Y, Zou Y, Ren J. Heavy metal scavenger metallothionein mitigates deep hypothermia-induced myocardial contractile anomalies: role of autophagy. Am J Physiol Endocrinol Metab 304: E74–E86, 2013. First published November 6, 2012; doi:10.1152/ajpendo.00176.2012.—Low-ambient temperature environment exposure increased the risk of cardiovascular morbidity and mortality, although the underlying mechanism remains unclear. This study was designed to examine the impact of cardiac overexpression of metallothionein, a cysteine-rich heavy metal scavenger, on low temperature (4°C)-induced changes in myocardial function and the underlying mechanism involved, with a focus on autophagy. Cold exposure (4°C for 3 wk) promoted oxidative stress and protein damage, increased left ventricular end-systolic and -diastolic diameter, and suppressed fractional shortening and whole heart contractility, the effects of which were significantly attenuated or ablated by metallothionein. Levels of the autophagy markers LC3B-II, beclin-1, and Atg7 were significantly upregulated with unchanged autophagy adaptor protein p62. Fluorescent immunohistochemistry revealed abundant LC3B puncta in cold temperature-exposed mouse hearts. Coimmunoprecipitation revealed increased dissociation between Bcl2 and Beclin-1. Cold exposure reduced phosphorylation of the autophagy inhibitory signaling molecules Akt and mTOR, increased ULK1 phosphorylation, and dampened eNOS phosphorylation (without changes in their total protein expression). These cold exposure-induced changes in myocardial function, autophagy, and autophagy signaling cascades were significantly alleviated or mitigated by metallothionein. Inhibition of autophagy using 3-methyladenine in vivo reversed cold exposure-induced cardiomyocyte contractile defects. Cold exposure-induced cardiomyocyte dysfunction was attenuated by the antioxidant N-acetylcysteine and the lysosomal inhibitor bafilomycin A1. Collectively, these findings suggest that metallothionein protects against cold exposure-induced cardiac anomalies possibly through attenuation of cardiac autophagy.

cold exposure; metallothionein; cardiac function; autophagy; autophagy flux

LOW-AMBIDENT TEMPERATURE ENVIRONMENT is known to increase the risk of cardiovascular morbidity and mortality through hypertension, stroke, and myocardial infarction (1–4). A 10°C drop in ambient air temperature is expected to trigger an approximate 15–20% rise in coronary events (7). Moreover, cold exposure seems to prompt much more pronounced cardiovascular sequelae in individuals with preexisting cardiovascular and respiratory illnesses (4, 17). Although a number of theories have been postulated for cold exposure-induced cardiovascular anomalies, including overactivation of endothelin-1 cascade, oxidative stress, and compromised antioxidant defense following cold stress (15, 26, 35), the ultimate culprit factor and effective therapeutic remedy against cold exposure-induced cardiovascular comorbidities remain elusive. Recent evidence from our laboratory revealed that cold exposure elicited cardiac geometric and contractile anomalies accompanied with overt oxidative stress, loss of mitochondrial biogenesis, and oxidative phosphorylation, the effects of which were alleviated significantly by knockout of endothelin-1 receptor (ETA) (35). Given that evidence from our group and others has consolidated a unique cardioprotective role of the heavy metal scavenger metallothionein in pathological conditions such as aging, obesity, insulin resistance, and diabetes mellitus (5, 8, 10, 11, 28, 29, 32), the present study was designed to examine the impact of cardiac-specific overexpression of metallothionein on low ambient temperature exposure (4°C)-induced myocardial contractile dysfunction, if any. Echocardiographic and Langendorff-perfused heart functions were assessed in adult wild-type friendly virus B (FVB) and metallothionein transgenic mice following cold stress exposure. Considering that autophagy, a regulated cellular process through which mammalian cells degrade and recycle macromolecules, organelles, and nutrients (20), is positively associated with cold exposure (30), essential protein markers of autophagy [LC3B, Atg7, beclin-1, and unc-51-like kinase (ULK1)] as well as the autophagosome cargo protein p62 were monitored in myocardium from FVB and metallothionein mice with or without low ambient temperature exposure. Fluorescent immunohistochemistry was used as an alternative approach for autophagy assessment (12). Immunoprecipitation technique was employed to evaluate the dissociation of beclin-1 from Bcl2, a process favoring autophagy induction (23). To explore the possible signaling mechanisms involved in autophagy regulation, both autophagy stimulatory and inhibitory signaling molecules, including AMP-dependent protein kinase (AMPK), Akt, and mammalian target of rapamycin (mTOR) (20, 31), were scrutinized. Levels of endothelial nitric oxide synthase (eNOS) and p70S6 kinase (p70S6K), pivotal cell signaling molecules governing cardiac homeostasis downstream of Akt, were evaluated. To evaluate the role of autophagosome formation and the late-stage autophagolysosome fusion (autophagy flux) in cold exposure-induced cardiac contractile anomalies, the effects of autophagosome inhibitor 3-methyl adenine (3-MA; in vivo and in vitro) and the lysosomal inhibitor bafilomycin A1 (in vitro) on cold stress-induced changes in autophagy and cardiac contractile function were tested. To understand the cause-effect relationship between oxidative stress and autophagy, the antioxidant N-acetylcysteine was used in cardiomyocytes from FVB mice with or without cold exposure.

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MATERIALS AND METHODS

Metallothionein transgenic mice, low temperature exposure, and drug treatment. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Wyoming (Laramie, WY). In brief, cardiac-specific, overexpressed metallothionein (driven by the mouse \( /H_9251 \)-myosin heavy chain promoter) mice were generated by crossing metallothionein heterozygous mice with wild-type FVB mice, as described previously (32, 33). The fur color was employed as a marker to identify metallothionein (dark brown) mice and their wild-type FVB littermates (white). Six-mo-old male metallothionein and FVB mice were housed at room temperature or low ambient temperature in a cold room (4°C) for 3 wk (6) within the School of Pharmacy Animal Facility with free access to food and water prior to assessment of myocardial morphology and function. To assess the effect of autophagy inhibition on cold-induced cardiomyocyte abnormalities, if any, FVB mice were maintained at room temperature or cold environment (4°C) for 3 wk with or without the autophagy inhibitor 3-MA (10 mg·kg\(^{-1}\), wk\(^{-1}\) ip) (9). To examine the role of oxidative stress in cold exposure-induced cardiomyocyte abnormalities, cardiomyocytes from FVB mice maintained at room temperature or cold environment (4°C) for 3 wk were incubated with the antioxidant N-acetylcysteine (NAC; 500 \( \mu\)M) for 4 h prior to evaluation of cardiomyocyte function (14). To further evaluate the role of autophagosome-lysosome fusion (autophagic flux) in cold exposure-induced cardiac contractile response, cardiomyocytes from FVB mice maintained at room temperature or cold environment (4°C) for 3 wk were incubated with the lysosomal inhibitor bafilomycin A1 (100 nM) for 4 h prior to evaluation of cardiomyocyte function.

Echocardiographic assessment. Cardiac geometry and function were evaluated in anesthetized (80 mg/kg ketamine and 12 mg/kg

Table 1. Information of antibodies

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ULK1, unc-51-like kinase; p, phosphorylated; mTOR, mammalian target of rapamycin; eNOS, endothelial nitric oxide synthase; AMPK, AMP-activated protein kinase.

Fig. 1. Effect of heavy metal scavenger metallothionein (MT) on low temperature (LT)-induced echocardiographic alterations. Friendly virus B (FVB) and MT mice were kept at normal (room) temperature (NT) or LT (4°C) for 3 wk prior to assessment of echocardiographic properties. A: body weight. B: heart rate. C: left ventricular (LV) wall thickness. D: LV end-systolic diameter (LVESD). E: LV end-diastolic diameter (LVEDD). F: LV mass. G: normalized LV mass. H: fractional shortening. I: cardiac output. Data are means ± SE; \( n = 9–10 \) mice/group. *\( P < 0.05 \) vs. FVB-NT group; #\( P < 0.05 \) vs. FVB-LT.
xylazine ip) mice using the 2-D guided M-mode echocardiography (Sonos 5500) equipped with a 15-MHz linear transducer at room or cold (4°C) temperature based on the mouse group assignment. Left ventricular (LV) anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M-mode using the methods adopted by the American Society of Echocardiography. Fractional shortening was calculated from LV end-diastolic (EDD) and end-systolic diameters (ESD) using the equation (EDD – ESD)/EDD. Heart rates were averaged over 10 consecutive cycles. Cardiac output was calculated using the equation (iTED – ESD)/EDD x heart rate (12).

Langendorff-perfused heart function. The Langendorff-perfused heart function was assessed using the ADInstruments PowerLab system. In brief, mice were anesthetized (80 mg/kg ketamine and 12 mg/kg ip xylazine), and hearts were perfused with KHB containing 7 mM glucose, 0.4 mM oleate, 1% bovine serum albumin, and a low level of insulin (10 μU/ml). The perfusion was initiated in the retrograde mode through the cannulated aorta. Hearts were perfused at a constant aortic pressure of 4 mmHg at baseline for 60 min. A fluid-filled latex balloon connected to a constant pressure transducer was inserted into the left ventricle to measure the pressure. LV-developed pressure (LVDP), the first derivative of LVDP, namely, the maximum rate of LV pressure development (+dP/dt) and the maximum rate of LV pressure decline (–dP/dt) were recorded using a digital acquisition system at a balloon volume that resulted in a baseline LV end-diastolic pressure of 5 mmHg (14).

Isolation of murine cardiomyocytes. After ketamine/xylazine sedation (80 mg/kg ketamine and 12 mg/kg ip xylazine), hearts were removed and perfused with Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 10 HEPES, and 11.1 glucose. Hearts were digested with Liberase Blendzyme 4 for 20 min. Left ventricles were removed and minced before being filtered. Myocyte yield was ~75%, which was not affected by low ambient temperature exposure or metallothionein transgene. Only rod-shaped myocytes with clear edges were selected for mechanical study (12).

Cell shortening/relengthening. Mechanical properties of cardiomyocytes were assessed using an IonOptix soft-edge system (IonOptix, Milton, MA). Myocytes were placed in a chamber mounted on the stage of an Olympus IX-70 microscope and superfused (~2 ml/min at 25°C) with KHB buffer containing 1 mM CaCl2. Myocytes were field-stimulated at 0.5 Hz. Cell shortening and relengthening, including peak shortening (PS), time to PS (TPS), time to 90% relengthening (TR90), and maximal velocities of shortening/relengthening (±d/dt), were assessed (12).

Fluorescent immunohistochemistry. Following anesthesia, hearts were excised and immediately placed in 10% neutral-buffered formalin at room temperature for 24 h after a brief rinse with PBS. Thereafter, heart tissues were dehydrated through serial alcohols and cleared in xylenes. The specimens were embedded in paraffin and cut into 5-μm sections. A cohort of samples was used for fluorescent immunohistochemistry to detect LC3B antibody expression and distribution. In brief, after the antigen unmasking by the citrate, sections were stained with the rabbit anti-LC3B antibody (1:500; Cell Signaling Technology), followed by incubation of the goat anti-rabbit IgG secondary antibody (Alexa Fluor 594; Invitrogen, Carlsbad, CA). Nuclei were counterstained with DAPI. Cardiac sections were visualized, and the ratio between LC3B-stained cell and nucleus was calculated on a digital microscope (×400) using the Image J (version 1.34S) and Photoshop software (13).

Reduced and oxidized glutathione. Tissue samples were homogenized and centrifuged. Supernatant fractions were collected for GSH and GSGG assay. One half of each sample was used for GSH determination and the other half for GSGG. Samples (100 μl) were incubated at room temperature with 2 μl of 4-vinyl pyridine for 1 h to conjugate GSH for determination of GSGG. The GSGG was then subtracted from the total glutathione to determine the GSH levels (24).

Intracellular reactive oxygen species measurement. Cardiomyocytes were loaded with 5-(6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate (1 μM) (Molecular Probes, Eugene, OR) for 30 min at 37°C for detection of intracellular reactive oxygen species (ROS). Cells were sampled randomly using an Olympus BX-51 microscope equipped with Olympus MagnaFire SP digital camera and ImagePro image analysis software (Media Cybernetics, Silver Spring, MD). Fluorescence was calibrated with InSpeck microspheres (Molecular Probes). An average of 100 cells was evaluated using the grid crossing method in 15 visual fields per isolation (34).

Protein carbonyl assay. Sample proteins were extracted from hearts. Then nucleic acids were eliminated by treating samples with 1% streptomycin sulfate for 15 min and centrifuged at 11,000 g for 10 min. Protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) to protein (0.5 mg). The TCA solution was removed, and samples were resuspended in 10 mM 2,4-dinitrope-

Fig. 2. Effect of heavy metal scavenger MT on LT-induced changes in myocardial contractility using the Langendorff-perfused heart system. FVB and MT mice were kept at NT or LT (4°C) for 3 wk prior to assessment of myocardial contractility. A: LV-developed pressure (LVDP). B: maximum rate of LV pressure development (+dP/dt) and C: maximum rate of LV pressure decline (–dP/dt). Data are means ± SE; n = 3–4 hearts/group. *P < 0.05 vs. FVB group; #P < 0.05 vs. FVB-LT group.
Fig. 3. Effect of heavy metal scavenger MT on LT-induced changes in myocardial oxidative stress [measured by reactive oxygen species (ROS) accumulation and GSH and GSSG levels] and autophagy. FVB and MT mice were kept at NT or LT (4°C) for 3 wk prior to assessment of oxidative stress and autophagy. A: representative fluorescent images (×400) showing ROS production using 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate in cardiomyocytes. B: pooled data of ROS production from 10 fields from 3 mice/group. C: GSH. D: GSSG. E: GSH/GSSG ratio. F: LC3B-II level. G: LC3B-II/LC3B-I ratio. H: Atg7 level. I: unc-51-like kinase (ULK1) level. J: phosphorylated (p)-ULK1 (Ser777) level. K: p-ULK1 (Ser777)/ULK1 ratio. F, H, I, and J, top: representative gel blots depicting autophagy protein markers (GAPDH as loading control) using specific antibodies. Data are means ± SE; n = 4–5 mice/group. *P < 0.05 vs. FVB-NT group; #P < 0.05 vs. FVB-LT group.
nylhydrazine solution prior to incubation at room temperature for 15–30 min. After addition of 500 μl of TCA, samples were centrifuged for 3 min. The supernatant was discarded, and pellets were washed in ethanol-ethyl acetate and allowed to incubate at room temperature for 10 min. The samples were centrifuged again for 3 min, and the ethanol-ethyl acetate steps were repeated twice. The precipitate was resuspended in 6 M guanidine solution and centrifuged for 3 min to remove any insoluble debris. The maximum absorbance (360–390 nm) of supernatant was read against appropriate blanks (water, 2 M HCl), and carbonyl content was calculated using the molar absorption coefficient of 22,000 M/cm (34).

Western blot analysis. Samples containing equal amount of proteins were separated on 10% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II; Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBST and incubated overnight at 4°C with anti-LC3B, anti-beclin-1, anti-Akt, anti-ULK1, anti-phosphorylated ULK1 (p-ULK1; Ser757), anti-Bcl2, anti-p62, anti-Akt, anti-phosphorylated Akt (p-Akt; Ser473), anti-mTOR, anti-phosphorylated mTOR (p-mTOR; Ser2448), anti-eNOS, anti-phosphorylated eNOS (p-eNOS; Ser1177), anti-AMPK, anti-phosphorylated AMPK (p-AMPK; Thr172), anti-p70S6K, anti-phosphorylated p70S6K (p-p70S6K; Thr389), and anti-GAPDH (loading control) antibodies (Table 1). After immunoblotting the film was scanned, and intensity of immunoblot bands was detected using the Bio-Rad Calibrated Densitometer. To avoid the potential impact of abrupt hemodynamic change, tissue collection was performed in a cold room for the low-temperature exposure groups (12).

Coimmunoprecipitation. The coimmunoprecipitation (Co-IP) assay was performed, following the protocol of the Pierce Co-IP kit. Briefly, 100 μg of purified beclin-1 antibodies were immobilized with coupling resin. Protein extracts (1,000 μg) were gently incubated with antibody-coupled resin, with end-over-end mixing for 120 min at room temperature. The resin was washed, and protein complexes bound to the antibody were eluted with 50 μl of elution buffer. The eluted protein was boiled at 95°C for 5 min and separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with Bcl2 and beclin-1 antibodies. Antibody binding was detected using the enhanced chemiluminescence. The membrane was scanned, and intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (model GS-800) (36).

Data analysis. Data were means ± SE. Statistical significance (P < 0.05) was estimated by a one-way analysis of variance followed by a Bonferroni multicomparison analysis.

RESULTS

Echocardiographic properties of FVB and metallothionein transgenic mice. Neither cold exposure nor metallothionein, nor both of them together, affected body weight, heart rate, LV wall thickness, LV mass (absolute or normalized value), or cardiac output. Cold exposure significantly increased LVESD and reduced fractional shortening, the effects of which were significantly attenuated or obliterated by metallothionein and metallothionein transgene itself did not elicit any notable effect on ROS production, carbonyl formation, or oxidative stress status in the absence of cold exposure (Figs. 3, A–E, and 4A). These findings favored an essential role of oxidative stress in

Effect of cold exposure on oxidative stress and autophagy in FVB and metallothionein mice. As expected, sustained cold exposure promoted ROS production and carbonyl formation as well as decreased levels of glutathione and the GSH/GSSG ratio without the levels of oxidized glutathione (GSSG) being affected, the effect of which was significantly attenuated by metallothionein. Metallothionein transgene did not elicit any effect on ROS production, carbonyl formation, or oxidative stress status in the absence of cold exposure (Figs. 3, A–E, and 4A). These findings favored an essential role of oxidative stress in...
cold exposure- and metallothionein-induced changes in myocardial contractile function.

To explore the possible mechanism(s) behind metallothionein transgene-induced protection against cold exposure, expression of autophagy markers LC3B, p62, beclin-1, Atg7, and ULK1 (total and phosphorylated Ser777 forms) was examined in FVB and metallothionein mouse hearts with or without cold exposure. Our results depicted that cold exposure significantly upregulated the levels of LC3B-II, LC3B-II/LC3B-I ratio, and beclin-1, Atg7, and ULK1 Ser777 phosphorylation (absolute or normalized value) without affecting levels of total ULK1 and the autophagy adaptor protein p62, indicating facilitated autophagy following cold exposure. Although metallothionein transgene itself did not affect the expression of these autophagy markers, it significantly attenuated or ablated cold exposure-induced elevation in LC3B-II, LC3B-II/LC3B-I ratio, and beclin-1, Atg7, and ULK1 phosphorylation without affecting other autophagy markers tested (Figs. 3, F–K, and 4, B and C).

Our further scrutiny of autophagy using the fluorescence immunohistochemistry revealed overt autophagy following cold exposure in murine hearts manifested as abundant LC3+ puncta. Similarly to the Western blot finding, metallothionein and autophagy inhibition using the autophagy inhibitor 3-MA (10 mg·kg⁻¹·wk⁻¹ ip for 3 wk) significantly alleviated a cold exposure-induced rise in LC3+ puncta without eliciting any overt effect by themselves (Fig. 5). These findings favored a possible role of autophagy induction in cold exposure- and metallothionein-induced changes in myocardial contractile function.

Western blot analysis of signaling molecules of Akt, AMPK, p70S6K, mTOR, and eNOS in FVB and metallothionein mice. To explore cell signaling mechanism(s) of cold exposure- and metallothionein-induced changes in myocardial autophagy, signaling proteins participating in autophagy regulation, including Akt, AMPK, p70S6K, mTOR, and eNOS, were examined. Our data revealed that sustained cold exposure reduced phosphorylation (absolute or normalized value) of the autophagy inhibitory signaling molecules, including Akt, mTOR, and eNOS (without affecting that of AMPK and p70S6K). Total protein expression of Akt, mTOR, eNOS, AMPK, and p70S6K was unaffected by cold exposure. Although metallothionein itself did not exert any effect on these autophagy regulatory proteins, it significantly attenuated or ablated cold exposure-induced changes in the phosphorylation of Akt, mTOR, and eNOS without affecting the responses of AMPK and p70S6K (Figs. 6 and 7).

Immunoprecipitation for autophagy through dissociation of the beclin-1 and Bcl2 complex. Beclin-1 (a BH3-only protein) binds to the BH3 domain of Bcl2, the conformation of which may be altered by phosphorylation in the BH3 domain, resulting in the release of beclin-1 from the complex and induction of autophagy (23). To examine the interaction between Bcl2 and beclin-1, immunoprecipitation was performed in FVB and metallothionein mouse hearts with or without cold exposure. Our result shown in Fig. 8A indicated that the amount of Bcl2 that immunoprecipitated with beclin-1 was significantly decreased in FVB mice exposed to low temperature. Although metallothionein transgene itself did not affect the ratio between Bcl2 and beclin-1, it removed cold exposure-induced dissociation between Bcl2 and beclin-1. The level of Bcl2 was unaffected by cold exposure, metallothionein, or both of them together. This observation favors the notion that metallothionein may protect against cold exposure-induced autophagy induction through reversing cold-triggered dissociation of Bcl2 and beclin-1.

Effect of autophagy inhibition on cold exposure-induced cardiomyocyte contractile anomalies. To consolidate a permissive role of autophagy induction in cold environment-induced cardiac contractile dysfunction, FVB mice maintained at room temperature or in a cold room (4°C) were treated with or
without the autophagy inhibitor 3-MA (10 mg·kg⁻¹·wk⁻¹ ip) for 3 wk prior to assessment of cardiomyocyte contractile function. Our data, shown in Fig. 8, revealed that cold exposure significantly depressed PS and ±d/dt and prolonged TR90 without affecting resting cell length and TPS in murine cardiomyocytes. Although 3-MA itself did not affect cardiomyocyte mechanics (except for the slightly reduced resting cell length), it significantly attenuated cold exposure-induced cardiomyocyte mechanical anomalies. These data favored a role of autophagy in cold exposure-induced cardiac contractile dysfunction.

**Role of lysosomal inhibition on cold exposure-induced cardiac contractile dysfunction.** Autophagy comprises essential steps of the early-stage autophagosome formation (inhibited by 3-MA) and the late-stage fusion of autophagosome with lysosome (inhibited by bafilomycin A1) (22). To evaluate the effect of autophagosome-lysosome fusion inhibition on cold exposure-induced cardiac contractile dysfunction, cardiomyocytes from normal temperature- and cold-exposed mouse hearts were incubated with the autophagic flux inhibitor bafilomycin A1 (100 nM) for 4 h in vitro prior to assessment of cardiomyocyte mechanics and autophagy protein markers. Our data revealed that bafilomycin A1 further exacerbated cold exposure-induced elevation in LC3B-II and LC3B-II/LC3B-I ratio and promoted p62 accumulation. Bafilomycin A1 did not affect autophagosome formation (LC3B-II and LC3B-II/LC3B-I ratio), although it interrupted autophagic flux (as evidenced by p62 accumulation). Similarly to the effect of 3-MA, bafilomycin A1 attenuated or ablated cold exposure-induced cardiomyocyte dysfunction, including depressed PS and ±d/dt and prolonged TR90. Bafilomycin A1 itself failed to significantly affect cardiomyocyte contractile properties (Fig. 9).
These findings favored a likely beneficial role of inhibition of autophagolysosome fusion against cold exposure-induced cardiac contractile dysfunction.

Effect of antioxidant on cold exposure-induced cardiomyocyte contractile anomalies. To explore the cause-effect correlation between oxidative stress and autophagy in cold exposure-induced cardiac contractile dysfunction, cardiomyocytes from FVB mice maintained at room temperature or in a cold room (4°C) for 3 wk were treated with or without the antioxidant NAC (500 μM) or 3-MA (10 mM, used as a positive control) for 4 h in vitro prior to evaluation of autophagy and cardiomyocyte contractile function. Our data revealed that NAC and 3-MA significantly attenuated or mitigated cold exposure-induced elevation in LC3B-II and LC3B-II/LC3B-I ratio without affecting p62 accumulation. Neither NAC nor 3-MA affected autophagosome formation (LC3B-II and LC3B-II/LC3B-I ratio) or autophagolysosome fusion (p62) in cardiomyocytes from mice maintained under normal temperature. Both NAC and 3-MA significantly attenuated or ablated cold exposure-induced cardiomyocyte contractile dysfunction, including depressed PS and ±dl/dt and prolonged TR90. Neither NAC nor 3-MA overtly affected cardiomyocyte contractile properties in mice maintained under normal temperature (Fig. 10).

DISCUSSION

The salient findings from our current study revealed that the cysteine-rich heavy metal scavenger metallothionein significantly attenuated cold exposure-induced cardiac contractile dysfunction, oxidative stress, and induction of autophagy. Cold stress-induced autophagy induction was supported by the accumulation of the autophagy markers LC3B-II, beclin-1, Atg7, and ULK1 phosphorylation, albeit with little changes in the levels of autophagy adaptor protein p62. Involvement of autophagy in cold exposure-induced cardiac contractile anomalies was further substantiated by the fact that autophagy inhibition using either 3-MA (suppressing early-stage autophagosome formation) or bafilomycin A1 (inhibiting late-stage autophagolysosome fusion) significantly alleviated cold stress-induced cardiomyocyte contractile dysfunction. These results...
collectively suggest a possible role of autophagy in the regulation of myocardial contractile function under cold exposure and, more importantly, the therapeutic potential of antioxidant and autophagy inhibition in cold stress-induced myocardial dysfunction.

Our data revealed that 3 wk of cold exposure increased LVEDD and LVESD significantly as well as reduced fractional shortening, consistent with our recent finding using a longer cold exposure duration (5 wk) (35). These findings received further support from the Langendorff-perfused heart function assessment, where 3 wk of cold exposure significantly depressed LVDP and $\pm dP/dt$. It is noteworthy that longer duration of cold exposure promotes cardiac hypertrophy (27, 34). In our hands, 3 wk of cold exposure led to changes in LVESD, LVEDD, and fractional shortening in the absence of cardiac hypertrophy and altered cardiac output. These findings seem to

Fig. 8. Effect of MT on LT (4°C, 3 wk)-induced change in the interaction of Bcl2 and beclin-1 as well as autophagy inhibition-elicited effect on LT-induced cardiomyocyte contractile dysfunction. A: Bcl2/beclin-1 ratio; representative gel blots of Bcl2 and beclin-1 using specific antibodies are shown at top. All protein expressions were normalized to GAPDH. IP, immunoprecipitation; IB, immunoblot. B–G: cardiomyocyte mechanics from FVB mice maintained at NT and LT for 3 wk with or without the autophagy inhibitor 3-MA (10 mg kg$^{-1}$·wk$^{-1}$ ip). B: resting cell length; C: peak shortening. D: maximal velocity of shortening ($+dL/dt$). E: maximal velocity of relengthening ($-dL/dt$). F: time to peak shortening (TPS). G: time to 90% relengthening (TR90). Data are means ± SE; $n = 123–126$ cells from 3 (B–G) or 3–4 mice/group (A). *$P < 0.05$ vs. FVB-NT group.
indicate that certain hemodynamic factors, such as pre- and after-load and ventricular wall compliance, may contribute to cold exposure-induced changes in myocardial contractile function. The apparent changes in both LVESD and LVEDD following cold exposure seem to favor presence of both systolic and diastolic dysfunction, although further scrutiny is needed to discern cardiac anomalies in systole and diastole. In addition, other scenarios may be considered for cold exposure-induced cardiac contractile dysfunction. For example, cold exposure has been reported to promote accumulation of ROS and oxidative stress as well as alteration of antioxidant defense (15, 35). This is supported by our findings of overt oxidative stress in cold-challenge hearts manifested as ROS accumulation, carbonyl formation, and reduced GSH/GSSG ratio. It is plausible to speculate that oxidative stress may play an essential role in cold exposure-induced cardiac anomalies, similar to...
Moreover, our immunoblotting and immunochemistry observations revealed induction of autophagy following cold exposure (shown as elevated LC3B-II, beclin-1, Atg7, and ULK1 phosphorylation as well as the beclin-1 dissociation from Bcl2), indicating a pivotal role of autophagy in cold exposure-induced cardiac dysfunction. This is further supported by the fact that autophagy inhibition using both 3-MA and bafilomycin A1 alleviated cold exposure-induced cardiomyocyte contractile dysfunction. Assessment of signaling mechanisms involved in autophagy regulation revealed that cold exposure-induced autophagy may be associated with dampened phosphorylation of Akt and mTOR, which are known to suppress autophagy (30). Autophagy is under the tight regulation of different upstream mediators, including the Atg (autophagy-related gene) family (such as Atg7 seen in our study) and mTOR kinase. The proteins encoded by Atg genes regulate the nucleation of autophagic vacuoles, formation of isolated double membrane (i.e., phagophore), maturation of autophagosome, and fusion with lysosomes forming autophagolysosome (24, 30). The other key regulator of autophagy is mTOR, the primary inhibitory molecule to turn off autophagy in response to various stimuli. Akt is perhaps the
most important upstream activator of mTOR to shut off autophagy. In contrast to Akt, AMPK inhibits mTOR phosphorylation and activates autophagy (24, 30). Data from our present study revealed reduced phosphorylation of Akt and mTOR following cold stress, favoring a role of lessened Akt/mTOR suppression on autophagy in cold stress-elicited autophagy induction. In our hands, neither expression nor phosphorylation of AMPK and p70S6K was altered significantly by cold stress, not favoring a role of AMPK (or p70S6K, which governs protein synthesis predominantly) activation in autophagy induction following cold stress. The unlikelihood of AMPK involvement in cold stress-induced autophagy induction is somehow inconsistent with the elevated ULK1 phosphorylation following cold stress. AMPK is known to promote autophagy by activating ULK1 at Ser317 and Ser777 phosphorylation sites (18). This finding suggests that cold exposure may induce autophagy through an AMPK-independent phosphorylation of ULK1. In addition, our finding of dampened eNOS phosphorylation following cold stress may or may not play a role in the regulation of autophagy. Although eNOS is known to impair autophagosome formation primarily via the Janus NH2-terminal kinase 1/Bcl2 pathway (25), it may directly regulate cardiac redox state and contractile function similarly to Akt and mTOR (19, 21). Nonetheless, the fact that autophagy inhibition using 3-MA or bafilomycin A1 reconciled cold stress-induced cardiomyocyte contractile dysfunction supports a permissive role of autophagy in cold stress-induced cardiac anomalies. Our data revealed that the lysosomal inhibitor bafilomycin A1 significantly attenuated cold stress-induced cardiomyocyte dysfunction through interrupting autophagy flux (as evidenced by LC3B-II accumulation and p62 buildup). This is distinct from that of 3-MA, which prevents autophagosome formation. Further study is warranted to better elucidate the interplay among Akt, mTOR, and eNOS in the regulation of autophagy and cardiac function following cold stress exposure.

Perhaps the most interesting finding from our study is that metallothionein overexpression alleviated cold stress exposure-induced cardiac remodeling, contractile dysfunction, oxidative stress, and autophagy induction. In addition to its ability to suppress oxidative stress, our data support the notion that the heavy metal scavenger may inhibit autophagy induction through restoring the loss of phosphorylation in Akt, mTOR, and eNOS under cold stress. Akt, mTOR, and eNOS signaling have been well defined to participate in the suppression of autophagy and oxidative stress as well as antioxidant-elicited cardioprotective benefits in pathological conditions (19, 21, 25). Autophagy usually starts with an “induction phase” that is initiated by beclin-1 as an internal stimulus, followed by a second “formation phase” involving Atg proteins such as Atg5, Atg7, and autophagosomal membrane-specific protein LC3 or Atg8, a marker for autophagosome. Eventually, autophagosome will fuse with lysosomes, using p62 as the autophagosome cargo protein, prior to degradation by lysosomal proteases (20, 31). Data from our study suggest enhanced autophagy in association with cardiac dysfunction and oxidative stress following cold stress, consistent with the notion that autophagy may be upregulated under environmental stress conditions (16). Our results strongly favor a role of autophagy in metallothionein-elicited cardioprotection, which is also supported by the finding that autophagy inhibition attenuated cold stress-induced cardiomyocyte contractile dysfunction. ROS regulates an array of signal transduction pathways to govern cell survival and death. In particular, ROS production such as under cold stress may activate autophagy and autophagic cell death (16). Our in vitro finding that NAC overtly attenuated cold stress-induced autophagy and cardiomyocyte contractile dysfunction favors a role of oxidative stress upstream of autophagy in cold exposure-induced cardiac anomalies. Further study is warranted to better understand the precise mechanism involved in the interplay between ROS and autophagy in metallothionein-elicited protection against cold stress.

In summary, data from our current study revealed that metallothionein overexpression rescues against cold exposure-induced myocardial contractile dysfunction. Our data favor the notion that oxidative stress and autophagy induction may play an essential role in cold stress- and metallothionein-elicited responses in cardiac mechanical function. Our findings further revealed a likely role of Akt, mTOR, and eNOS signaling in cold stress- and metallothionein-induced regulation of myocardial autophagy and contractile function. These data should shed some lights toward the therapeutic promises of antioxidants and autophagy regulators in cold stress-induced cardiovascular anomalies.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
S.J., R.G., and Y. Zhang performed the experiments; S.J., R.G., Y. Zhang, and J.R. analyzed the data; S.J., R.G., Y. Zhang, and J.R. interpreted the results and drafted the manuscript.

REFERENCES
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