Chronic cardiac structural damage, diastolic and systolic dysfunction following acute myocardial injury due to bromine exposure: Role of PLN

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Abstract

Background: Highly reactive halogens such as bromine are more commonly utilized, leading to an increase in quantities that are stocked and transported, therefore increasing the risk of accidental or deliberate exposure. Here we describe progression and potential mechanisms of adverse effects of a single injurious insult on the heart caused by bromine inhalation. Our studies have shown that bromine inhalation causes acute myocardial ischemia-reperfusion-like injury mediated by cytosolic calcium overload and increased calpain activity due to inactivation of SERCA. However, the progression of this injury in survivors is unknown. Our working hypothesis is that the initial injury causes irreversible damage that leads to chronic left ventricular systolic and diastolic dysfunction.

Methods: Sprague Dawley rats received bromine exposure of 600 ppm for 45' and the survivors were sacrificed at 14 or 28 days, with matching naïve groups at each time point. Echocardiography, hemodynamic analysis, histology, electron microscopy and biochemical analysis of cardiac tissue were performed to assess functional, structural and molecular effects.

Results: At 14 and 28 days, hemodynamic and echocardiographic analysis revealed increases in RV and LV end-diastolic pressure and LV end-diastolic wall stress with increased LV fibrosis at 28 days. TEM images demonstrated myofibrillar loss, cytoskeletal breakdown and mitochondrial damage at both time points. The myofibrillar damage and increased LV wall stress was reflected by increases in cTnI and NT-proBNP at both time points. LV shortening decreased as a function of increasing LV end-systolic wall stress and was accompanied by increased SERCA modification and a striking dephosphorylation of phospholamban with a significant increase in protein phosphatase 1. There was an increased 4-hydroxynonenal content in the myocardium at 28 days suggesting increased oxidative stress.

Conclusions: These results indicate that the initial insult of bromine inhalation initiates a continuous process with chronic myocardial damage and subsequent LV systolic and diastolic dysfunction and that oxidative stress and phospholamban dephosphorylation play a central role.

Introduction

Exposure to toxic gases such as bromine (Br_2) can result in significant morbidity and mortality[1]. Our limited understanding of the pathophysiology stems from few case reports of victims of accidental Br_2 inhalation demonstrating respiratory and myocardial injury, cardiac arrest and circulatory collapse [1, 2]. To overcome this barrier, studies utilizing animal models for Br_2 inhalation are emerging [3-13]. We have established that inhalation of halogens (chlorine or bromine) causes acute ischemia-reperfusion-type injury to the heart with biventricular dysfunction[12, 14]. These acute cardiac effects may persist in survivors and cause severe complications. However, there is a big gap in understanding of the long-term effects and progression of disease in such individuals.

Once inhaled, as with other halogens, Br_2 reacts with the moist airway surface forming highly bioactive brominated intermediates (brominated fatty acids and brominated fatty aldehydes)[5, 14]. Halogenated reactants reach the heart and react with important cardiac proteins such as SERCA2, modifying and inactivating it, causing a cytosolic Ca^{2+} overload, ATP depletion, decrease in mitochondrial transmembrane gradient and calpain activation[14, 15]. Modification of SERCA2 and Ca^{2+} overload is a central pathophysiological mechanism of various cardiac diseases, including cardiac hypertrophy and heart failure[11, 12, 14, 15]. We have shown that acute Br_2 inhalation inactivates SERCA2 resulting in increased Ca^{2+}-sensitive LV calpain activity [14]. Calpain activation causes degradation of myocardial contractile proteins such as titin and the major cytoskeletal protein desmin, resulting in cardiac contractile dysfunction[14, 15].

Cardiac SERCA2 is regulated by a key molecule called phospholamban (PLN), phosphorylation of which increases calcium uptake into the sarcoplasmic reticulum by increasing SERCA activity[16]. PLN phosphorylation inactivates SERCA2 by releasing itself from the

SERCA2 molecule. Phospholamban forms mutimeric protein complex to interact with SERCA2 and endoplasmic reticulum stress signaling molecules [17]. The fine tuning of PLN phosphorylation to regulate SERCA is carried out by binding of anchoring subunits of protein phosphatase 1 (PP1) and protein kinase A to PLN [17]. Perturbation of these networks of signaling molecules may lead to altered calcium cycling and cell death and is emerging at the forefront of cardiac disease pathogenesis [18, 19]. In this study we demonstrate that a single Br_2 exposure causes chronic cardiac hypertrophy, ultrastructural damage, myocardial remodeling and biventricular dysfunction in survivors. We also highlight mechanisms wherein oxidative stress, decreased PLN phosphorylation and increased PP1 plays a significant role.

Methods:

In vivo exposures to Br_2:

All animal procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Un-anesthetized male Sprague Dawley rats (200-250 g, Envigo, Indianapolis, Indiana) were exposed (whole body) to 600 ppm Br_2 for 45 min as described previously[3, 14, 20]. Rats were then returned to room air and monitored continuously up to 8 h, again at 24 h and at 48 h for clinical scores[14, 21]. Clinical scoring is a composite score of activity and respiratory quality as previously described[14, 21]. Oxygen saturation and heart rates were also monitored using the MouseOX small animal oximeter (Starr Life Sciences, Oakmont PA) as previously described in our laboratory[12, 14]. Animals were monitored until 14- or 28-day endpoint was reached.

At experimental endpoint surgery was performed after echocardiographic and hemodynamical analysis. Blood was collected from the descending aorta and arterial blood gases (ABG) were measured using the EPOC automated blood-gas analysis reader (Ottawa, ON, Canada)[12]. Plasma was analyzed for cardiac injury markers hFABP, NT-proBNP and cTnI by ELISA[12]. Bronchioalveolar lavage fluid [22] was collected, and the right upper lobe of the lung was isolated and collected, weighed for a wet weight measurement and then dried at 98 °C for 48 h, then weighted for a dry weight measurement in order to do a wet weight to dry weight ratio (WW/DW)[23]. Heart was collected and weighed in order to obtain heart weight to body weight ratio (HW/BW)[24], then the upper third was fixed in neutral buffered formalin for later histological processing and analysis. The apex was discarded and the mid-section was frozen for use in collagen quantification Assay.

Transthoracic Two-dimensional Echocardiography/Doppler (ECHO/Doppler) and RV and LV hemodynamics:

All experiments were performed under 2% isoflurane anesthesia in compressed room air as previously performed in our laboratory[25]. The body temperature was maintained at 37°C during measurements. Transthoracic two-dimensional ECHO/Doppler was performed using a Vevo2100 high- resolution ultrasound system (VisualSonics Inc., Toronto, ON, Canada) as previously described in our laboratory. Parasternal long- and short-axis two-chamber M-mode, and B-mode views were obtained at mid papillary level and averaged to determine LV dimensions at end-systole and end-diastole. LV volumes, cardiac output (CO), fractional shortening (FS), and ejection fraction were calculated (Visualsonics software). Spectral Doppler was used to determine trans-mitral early (*E*) and atrial (*A*) wave peak velocities. Operators blinded to exposure performed image collection and analyses. A polyethylene catheter (PE-50) was placed in the left jugular vein and a 2 F high-fidelity catheter (SPR-407, Millar Institute, Houston, TX, USA) was inserted into the LV via the right carotid artery. LV and RV high-fidelity pressures were measured with a Harvard data acquisition system interfaced with a PC with AcqKnowledge III (ACQ 3.2)[12, 14].

Collagen Analysis:

Hydroxyproline assay was performed using 10 mg of left ventricle and following instructions for Hydroxyproline Assay Kit (MAK008, Sigma-Aldrich)[26], and normalized by wet tissue weight.

Histological Imaging and Transmission Electron Microscopy (TEMs):

Tissue was obtained at terminal surgery and processed for imaging following previously described methods, using both H&E and PSR staining [14]. Images were obtained by using a 20× objective (600× on the video screen) of an Olympus AH3 research microscope with a monochrome

video CCD72 camera interfaced to a computer equipped with an Image One (Universal Imaging) morphometry system[27]. TEMs were performed as previously described (EMLabs, Birmingham Al) [28, 29].

Immunohistochemistry

Rat hearts were immersion-fixed in 10% neutral buffered formalin and paraffin-embedded. 5μm sections were mounted on + slides, deparaffinized in xylene and rehydrated in a graded series of ethanol. HIER was performed with citrate buffer (Vector Laboratories, #H-3300, Burlingame, CA). Sections were blocked with 5% goat serum (in 1% bovine serum/PBS), followed by overnight incubation at 4°C with primary antibody (4-Hydroxynonenal, Abcam #ab46545, Cambridge, MA, 1:100; Myosin, DSHB #MF20, University of Iowa Hybridoma Bank, 1:10). Sections were incubated with Alexa Fluor 488- or 594-conjugated secondary antibody (Life Technologies/Molecular Probes, Eugene, OR, 1:700) to visualize lipid peroxidation products and cardiomyocyte structure, as indicated in figure legends. Nuclei were stained with DAPI (1.5μg/ml, Vector Laboratories #H-1500). Image acquisition was performed on a Leica DM6000 epifluorescence microscope with SimplePCI software (Compix, Inc., Cranberry Township, PA). Images were adjusted appropriately to remove background fluorescence.

Immunoprecipitation and immunoblots

Rat hearts lysates (500 mg protein) were immunoprecipitated for SERCA2 using monoclonal mouse antibody (2 μg/ml, Thermo Scientific) with 20 μl protein G magnetic beads (Cell Signaling Technology). Immunoprecipitate protein captured on the beads was eluted in the sample buffer and resolved on polyacrylamide gels for subsequent Western blotting using SERCA and Br-Tyr antibody as described previously[14]. Immunoblots on cardiac tissues were performed according to previously described methods using antibodies against SERCA2 (1:1000 abcam), Br-

Tyrosine (1:1000; JaICA), phospho-phospholamban (1:1000, Cell Signaling Technology), phospholamban (1:1000, Cell Signaling Technology), PP1 (1:1000 R&D Systems), NOX4 (1:500 Novus Biologicals) and GAPDH (1:5000, Cell Signaling Technology), Hydroxynonal (1:1000 abcam).

Statistical Analysis:

Data are expressed as Mean±Standard Error analyzed by one-way analysis of variance (ANOVA) with Student's paired test. A p value <0.05 was considered significant. Analysis was conducted using Graphpad Prism version 7 software (LaJolla, CA). All echocardiography analysis and calculations were performed using SPSS version 19.0 (SPSS, Inc., Chicago, IL). Two observers with expertise in echocardiography assessed the studies for intra- and inter-observer reproducibility.

Results:

Increase in Cardiac Mass and Myofibrillar breakdown

Figure 1A demonstrates an increase in circulating NT-proBNP at 14 and 28 days with values of 44±5 and 19±6 pg/ml in Br_2 exposed rats, respectively, compared to undetectable levels in naïve animals. This increase in NT-proBNP was supported by a significant increase in heart weight by body weight ratio (HW/BW) at the 14-day (0.0037 ± 0.00007 HW/BW, $p<0.05$) and 28 day (0.0035 ± 0.00005 HW/BW $p<0.05$) compared to naïve 0.0031 ± 0.00005 HW/BW ratio (**Figure 1B**). This is also supported values of LV volumes and LV mass determined by echocardiography (Supplementary **Table 1**), demonstrating a statistically significant increase in the 28-day LV mass (1010 ± 29 mg $p<0.05$) in Br_2 exposed rats compared to naïve rat LV mass (856.0 ± 21.3 mg).

We have previously reported severe myofibrillar loss and mitochondrial damage and an increase in cardiac injury markers at 24 h and 7 days after an exposure to 600 ppm of Br_2 for 45 minutes. In this study at 14- and 28-day post Br_2 exposure we observed a persistent marked myofibrillar breakdown along with mitochondrial damage and disorganization and disruption of the sarcomeric and mitochondrial structure (**Figure 2**). In addition, **Figure 2C** demonstrates a near five-fold increase in circulating troponin levels at 14 and 28 days (5.2 ± 1.6 ng/ml and 3.3 ± 0.9 ng/ml), compared to naïve controls (1.4 ± 0.04 ng/ml $p<0.05$).

Extracellular Matrix Pathology

PSR stained cardiac histological images demonstrated an increase in spacing between perimysial myofiber bundles that disrupts the laminar structure throughout the endocardium and mesocardium at the 14-day timepoint (**Figure 3B and 3E**) and at the 28-day timepoint (**Figure 3C and 3F**) compared to the naïve group (**Figure 3A and 3D**). This breakdown of the larger

laminar structure of the LV was previously observed at 2 and 7 days and as demonstrated here persists at 14 and 28 days, most likely represents myocardial edema[14]. However, TEM images demonstrated marked increase in extracellular endomysial fibrillar collagen (**Figure 4a-f**). Analysis of LV hydroxyproline content demonstrates an increase at 14 days (0.587 ± 0.058) that achieves statistical significance at 28 days (0.770 ± 0.032 $p<0.05$) compared to naïve rats (0.502 ± 0.062) (**Figure 4c**). This supports both persistent edema as well as fibrosis in the hearts at the 28-day timepoint after Br_2 inhalation. We also investigated if there was pulmonary injury and edema present in these animals at these later timepoints. As opposed to the hearts, there was no significant difference in BALF protein content (an indicatory of lung injury) in 14-day (0.12 ± 0.01 mg/ml, $p=0.129$) or 28-day (0.15 ± 0.02 mg/ml, $p= 0.143$) after bromine exposure in exposed rats as compared to naïve rats (0.18 ± 0.02 mg/ml). In addition, lung WW/DW ratio (an indicator of pulmonary edema) did not differ in 14-day (4.91 ± 0.14) or 28-day (4.77 ± 0.05) after bromine exposure in exposed animals as compared to naïve rats (4.84 ± 0.02).

Hemodynamic and LV functional Analysis

Mean arterial blood pressure was increased at both 14 and 28 days post Br_2 exposure (101 ± 3 mmHg, $p<0.05$ and 100 ± 2 mmHg, $p<0.05$) compared to naïve rats (88 ± 3 mmHg) (Supplementary **Table 1**). RVEDP and RV peak pressure increased only at the 28-day time point compared to naïve (1.25 ± 0.40 mmHg to 3.20 ± 0.36 mmHg ($p<0.05$) (Supplementary **Table 1**). LVED pressure and LVED wall stress increased significantly at 14-day and 28-day time points that culminates into a statistically significant increase in LVEDV at 28 days post bromine exposure (Supplementary **Table 1**). Sphere index, LV relative wall thickness measured from LVED dimension and LV wall thickness and LV Mass/LVEDV ratio are unchanged compared to naïve rat values (Supplementary **Table 1**).

There was also a significant increase in both LV end-systolic volume and end-systolic wall stress at 28 days coupled with a decrease in LV fractional shortening with a trend toward a decrease in VcFr at 28 days (**Figure 5A and 5B**). To further analyze the relationship between LVES wall stress and LV rate of shortening other parameters were evaluated. A significant decrease in the VcFr/ESWS ratio at both 14-day (0.11 ± 0.01, $p < 0.05$) and the 28-day (0.12 ± 0.01, $p<0.05$) time points after Br_2 exposure as compared to naïve naive rats (0.164 ± 0.016). Increasing LV end-systolic wall stress also correlated with increasing LVESV and LVESD that translated to a corresponding significant correlation of increasing LVES wall stress with decreasing VCFr and FS (**Figure 5C-E**). Taken together, the adverse systolic remodeling of the heart with underlying myofibrillar loss is associated with progressive decrease in both rate and extent of LV shortening.

Calcium Handling Proteins and oxidative stress

In conjunction with the decrease in contractile function, we also observed significant increase in cardiac SERCA modification (bromination of tyrosine residues) and decreased expression 28 days after bromine inhalation (**Figure 6A and 6B**). Quantitation of the Westerns revealed increased brominated SERCA2/SERCA2 ratio in the hearts of animals 28 days after exposure as compared to naïve animals ($p<0.01$), however at 14 days after exposure it was not significantly elevated (**Figure 6B**). Our previous study demonstrated an early increase in the protein content of phospholamban an important SERCA regulator[14]. We evaluated both total phospholamban and phospholamban phosphorylation (Ser16/Thr17) in the hearts of the bromine exposed animals. A dramatic loss of phospholamban phosphorylation in the LV of both the 14 day and in the 28-day group compared to naïve animals was observed (**Figure 6C and 6D**) without a change in total phospholamban expression (**Figure 6C and 6E**). Protein phosphatase 1, PP1, the major phosphatase of phospholamban trended towards an increase at 14 days and achieved a near

three-fold increase at 28 days (**Figure 6F and 6G**). As NOX4 plays a significant role in the activation of PP1, we evaluated its expression and found no change in the 14-day or 28-day group compared to the naïve group (**Figure 6F and 6H**). Another factor that induces PP1 is oxidative stress [19]. Myocardial hydroxynonenal (HNE) accumulation and subsequent protein modifications are known to contribute largely to cardiac and vascular disease pathology [30]. Therefore, to understand the basis of these underlying effects of a single bromine exposure we evaluated the 4-HNE content in the hearts of animals 14- and 28d-after bromine exposure. A significant increase in the 4-HNE content was observed in the bromine exposed hearts (at 14- and 28- day timepoint post bromine exposure) as compared to naïve animals (**Figure 7A and 7B**). Immunohistochemistry, IHC, was performed for 4-HNE and myosin. A significant increase in HNE was observed and the staining was primarily located intracellularly in exposed animals compared to extracellularly in naïve animals (**Figure 7C**). Furthermore, normal myofibrillar structure was shown to be present in naïve animals, while in animals at 14 days after exposure, cardiomyocytes also presented loss of normal myofibrillar structure, and at 28 days cardiomyocytes that have lost their nuclei and myofibrillar structure indicating necrosis were observed (**Figure 7C**). Thus, our results demonstrate that the survivors of a single bromine inhalation event may have significant cardiac dysfunction due to increased cardiac hypertrophy and stress caused by oxidative stress and perturbations in important cardiac function regulators in vasculature, intracellular and extracellular compartments in the heart (**Figure 8)**.

Discussion:

Halogens such as bromine are produced, transported and stored in large quantities to be used in various industries all over the United States which is one of the top global halogen gas producers. Besides being an occupational hazard for the factory employees, the increased production and abundance further enhances the risk of accidental or intentional exposure to mass populations. Therefore, there is a growing need to understand the mechanisms and nature of injuries caused by exposures to these toxic gases to invent potential therapeutic strategies and educate clinicians and public health personnel. We have previously reported acute myocardial injury with significant ultrastructural changes leading to biventricular cardiac dysfunction after Br_2 inhalation. Here we demonstrate the persistence of myocardial pathology with hemodynamic and functional evidence of heart failure in survivors of a single Br_2 inhalation incident. These results for the first time demonstrate the long-term effect on cardiac function in survivors of a single Br_2 inhalation. Moreover, any acute cardiac event can lead to recurrence of myocardial infarction, stroke or cardiovascular death but the mechanisms and predictors of such adverse progressions are unknown. This mechanistic study is designed to understand such a pathogenic process.

There is also very sparse clinical information regarding the heart in humans exposed to halogens. Cardiomegaly has been reported on autopsy in 8 of 9 victims of acute chlorine poisoning in the 2005 South Carolina train derailment [31-33]. Other reports have also described cardiomegaly in association with pulmonary edema and vascular congestion of the lungs, liver, and other organs. Lung congestion and pulmonary edema may explain subsequent RV hypertrophy and dilation; however, these case reports provide no insight into whether cardiomegaly results from primary RV and LV injury or a combined cardiopulmonary process. Our studies in acute and chronic Br_2

exposure now demonstrate acute and chronic cardiac injury that is independent and separate from lung injury[14].

Cardiac hypertrophy has been linked to progressive systolic disfunction and other cardiac morbidities [34-37]. Most of the victims that died due to accidental chlorine exposure had cardiomegaly as revealed in the autopsy reports [38]. Toxic inhaled gases such as halogens and carbon monoxide may cause cardiac hypertrophy on one hand and inhalation of other gases such as nitric oxide and hydrogen may help reverse hypertrophy caused by other agents/mechanisms [39, 40]. Persistent increase in troponin and NT-proBNP is a great prognostic value for predicting serious cardiac outcomes in patients [41, 42]. These key circulating heart-specific biomarkers (brain natriuretic protein, BNP and troponin) were elevated at both 14 and 28 days. This is consistent with the increased in LV diastolic and systolic wall stress and heart weight/body weight in addition to the extensive myofibrillar breakdown by TEM, respectively. These same changes were present acutely in rats exposed to Br_2 and persist at 28 days after exposure. There also is a persistence of a fractured laminar structure of the heart manifested by the spaces between myocyte bundles. Numerous studies in the rat report a very organized laminar orientation of myofibers changing in direction from -70 to +70 form endo-to mesocardium. The orderly connection of this laminar structure is a necessary feature of LV contractile function and electrical propagation. At the same time, there is extensive collagen accumulation between individual cardiomyocytes by TEM that is verified by a significant increase in hydroxyproline. These early and continuous global damage can account for the diastolic dysfunction manifested by elevation of RV and LV filling pressures and LV diastolic wall stress.

LV wall stress is a sensitive mechanical marker of cardiac hypertrophy with preserved ejection fraction in hypertrophic cardiomyopathy patients, however, it needs to be very carefully evaluated and interpreted as its role in cardiac function and pathology is still being explored [43, 44]. It is also difficult to measure clinically and is interpreted via surrogate measures such as systemic vascular resistance via a pulmonary artery catheter. However, LV wall stress may not be the only contributor to the increased NT-proBNP, although higher levels of this peptide were found to correlate with mortality in patients with heart failure[45].

At the cardiomyocyte level, in addition to significant myofibrillar breakdown, there is complete disorganization of the sarcomere with breakdown of the z disc and disarray and proliferation of mitochondria. This pattern is also prominent in the acute state of Br_2 exposure in the rat and persists in the chronic state. In combination with this severe ultrastructural damage is a marked de-phosphorylation phospholamaban (PLN) at 14 and 28 days coupled with a significant increase in PP1 leading to SERCA2 inactivation. Reduced SERCA2 expression and hypophosphorylated PLN are important contributors in impaired Ca^{2+} handling and reduced contractibility of failing cardiomyocytes [46]. Further inactivation of SERCA derives from molecular modifications, such as bromination generating Br-SERCA, which in this case is increased when corrected by total SERCA in animals 28 days after exposure. Although, we did not find measurable amounts of brominated fatty acids in cardiac tissues in delayed samples the halogenation of SERCA at this later time point could be caused by a later accumulation of other brominated moieties or from the hypochlorous or hypobromous acids (HOCl or HOBr) formed by myeloperoxidase activity in vivo[47, 48].

These changes are pathognomic of the failing human heart; however, the stimulus for this critical regulation of contractile function resulting from acute Br_2 exposure is an open question.

LV NOX4, and important regulator of PP1 regulation, protein expression at 14 and 28 days post Br_2 exposure is unchanged compared to naïve rats. PP1 is also activated by increases of oxidative stress, in our model we can observe increased oxidative stress by the increase in HNE expression. Oxidative stress plays a critical role in pathological cardiac remodelling [49]. PP1 phosphorylates PLN at both Ser-16 and Thr-17 residues. Other phosphatases such as PP2Ce that phosphorylate only the Thr-17 of PLN were found to be increased in patients with cardiomyopathy and its expression cause decreased contractility and oxidative injury and susceptibility to ischemia-reperfusion injury in transgenic mice[50].

The persistent whole heart, ultrastructural, and biochemical changes coupled with progressive systolic and diastolic dysfunction support the contention that acute Br_2 exposure causes severe irreversible damage to the myocardium. There is a significant increase in both LV end-systolic volume and end-systolic wall stress at 28 days coupled with a decrease in LV fractional shortening with a trend toward a decrease in VcFr. In an attempt to connect LV remodeling with LV systolic function, and Figure 5 demonstrates the correlation of LVES volume, LV fractional shortening, and VCfr with increasing LV end-systolic wall stress, consistent with the striking dephosphorylation of phospholamban and significant increase in protein phosphatase. As of now, there is no knowledge on how the survivors of acute bromine inhalation would progress with their underlying cardiac injury. When one takes into consideration the hypertrophy, edema, fibrosis, increased cardiac injury markers, ventricular dilation, decreased contractility, diastolic and systolic dysfunction, we conclude that there is an ongoing deterioration and early stages of heart failure. Moreover, we demonstrate a marked cardiac PP1 dependent PLN hypo-phosphorylation post bromine exposure which is a molecular hallmark of heart failure[18, 51].

Figure Legends:

Figure 1: Survivors of bromine exposure have persistent myocardial remodeling and cardiac hypertrophy. Rats were exposed to 600 ppm Br_2 for 45 minutes and transferred to room air. Surviving rats were sacrificed at 14 or 28 days after exposure and blood was collected from the descending aorta. NT-proBNP (A) and Heart weight to body weight ratios (B) were increased significantly at both time points compared to age-matched naïve controls. Data shown are mean±SE (n=6-11 for each group), *indicates $p<0.05$ compared to naïve controls.

Figure 2: Br_2 inhalation causes persistent disruption of cardiomyocyte cytoskeleton and loss of the normal highly organized linear mitochondrial-sarcomere integrity. As described in Figure 1 rats were exposed Br_2, transferred to room air, and surviving animals were sacrificed and cardiac tissue collected at 14- and 28-day time points and fixed for transmission electron microscopy (TEM). Representative TEM of control (4000X a and b) and Br_2 exposed rats (13000X d-f)) Br2 exposed rats have extensive myofibrillar loss (yellow arrows) and disruption of z-discs (yellow arrowheads) in addition to mitochondrial swelling, cristae lysis and extensive mitochondrial vacuolization (red asterisks). Aortic blood troponin I, cTnI, was increased significantly at both time points (c). Data shown are mean±SE (n=6 for each group), * indicates $p<0.05$ vs unexposed control (naïve).

Figure 3: PSR staining for collagen in rat hearts after Br_2 exposure. As described in Figures 1 and 2, 14- and 28-days post Br_2 exposure cardiac tissue was fixed and embedded in paraffin and stained with picric acid sirius red (PSR). Images at 1X magnification for naïve group and Br_2 groups demonstrate, ventricular cavity [52], endocardium (2) and mesocardium (3). Control LV demonstrates a compact myocardium while the images from 14 d group or the 28 d groups show loss of continuity in the endocardium and increase in spacing between myocardial muscle fibers (arrows). 20X images (bottom panels) of mid myocardium demonstrates diffused fracturing and increased interstitial space in the 14 d and 28 d group (arrows). Arrow heads indicate interstitial collagen fiber deposition in the 14 and 28 d groups.

Figure 4: Br_2 inhalation causes myocardial remodeling and fibrosis. Representative TEM (4,000X (a and b) and 13,000X (d-f)) images demonstrate marked collagen fiber deposition in the interstitium (red arrows) of the Br_2 exposed rat hearts at 28 days (28 d). LV hydroxyproline content (c) at 28 days was significantly increased compared to controls. Data shown are mean±SE (n=5-6 for each group), * indicates $p<0.05$ vs unexposed control.

Figure 5: Br_2 inhalation increases left ventricular wall stress. At 14 and 28 days after exposure LV high-fidelity pressure and echocardiography was obtained under isoflurane anesthesia. LV end-systolic wall stress (A) was increased at 28 days, while VCFr/ESWS (B) was decreased at both time points. Linear regression with quadrant representations demonstrate the relation between LV end-systolic wall stress to VCFr (C), ESV (D), FS (E) and LVESD (F) with 95% confidence intervals. Mean±SE values for the naïve group were used to make quadrants (dotted lines). Data shown are mean±SE (n=8-14 for each group), * indicates $p<0.05$ vs. naïve controls.

Figure 6: Mechanisms of Br_2 exposure-induced myocardial SERCA2 modification. (A-B) Role of chemical modification: Lysates were prepared from the LV of naïve or bromine exposed

rats and immunoprecipitations were performed using 1 μg/ml anti-rat SERCA2 antibody and 500 μg protein lysate. Western blots were performed using immunoprecipitated proteins separated by magnetic beads as described in the Methods. Antibodies against Br-Tyrosine (Br-Tyr) and SERCA2 (representative blots shown in the top and middle panel) were used to determine SERCA2 modification and SERCA2 expression. IgG released from the beads was used as a loading control. Values are expressed as Br-SERCA/SERCA ratio, and corrected by IgG (B). Data shown are mean±SE (n=4 for each group), * indicates $p<0.05$. (C-E) **Role of Br_2-induced loss of phospholamban phosphorylation in the myocardium.** Left ventricle of naïve rats or of the rats exposed to bromine 14 or 28 days before were collected and lysates were prepared for Western blots as described in the Methods. Antibodies against anti rat phospho-phospholamban (P-PLN), phospholamban (PLN) were used at a dilution of 1:1000. GAPDH expression was used as a loading control. Representative blots of at least two reproducible experiments are shown. Data are mean±SE (n=4 for each group), * indicates $p<0.05$ as compared to controls. (F-H) **Br_2 inhalation causes increased protein phosphatase (PP1) expression in the myocardium.** Left ventricle of naïve rats or of the rats exposed to bromine 14 or 28 days before were collected and lysates were prepared for Western blots as described in the Methods. Antibodies against protein phosphatase 1 (PP1) and NADPH oxidase 4 (NOX4) were used at a dilution of 1:1000. GAPDH expression was used as loading control. Representative blots of at least two-three reproducible experiments are shown. Data shown are mean±SE (n=4 for each group), * indicates $p<0.05$ as compared to naïve control.

Figure 7: Bromine exposure induces increased cardiac oxidative stress in survivors. Left ventricle of naïve rats or of the rats exposed to bromine 14 or 28 days before were collected and lysates were prepared for Western blots as described in the Methods. Antibodies against 4-

hydroxynonenal (HNE) were used at a dilution of 1:1000. GAPDH expression was used as a loading control (A). Values are expressed in arbitrary units (AU), and corrected by GAPDH (B). Data shown are mean±SE (n=5-6 for each group), * indicates $p<0.05$ vs. naïve control. **C) HNE localization and myosin structural changes in the hearts of Br_2-exposed rats.** Immunohistochemistry was performed for hydroxynonenal (HNE) (red), myosin [53] [53]and DAPI as a nuclear stain (blue). HNE staining as shown in panels for 14 and 28 d groups demonstrates increase in oxidative stress (more obvious in the sample collected 28 days after exposure). Myosin + DAPI staining shows conserved myofibrillar structure in naïve group (red arrows) fibers that are in degradation process (white arrows) and cardiomyocytes that have gone through necrosis cells (white arrowheads) in 14 or 28 d post Br2-exposed groups. In the overlay one can observe the location of HNE as compared to myocardial structures.

Figure 8: Schematic representation of mechanisms of delayed Br_2 induced cardiac stress, dysfunction and remodeling leading to heart failure in survivors. High concentrations of bromine inhalation cause deaths in the victims due to cardiopulmonary damage. The survivors have continuous release of cardiac damage markers in the circulation and increased edema and fibrosis in the heart. These could be results of increased oxidative stress in the myocardium causing a vicious cycle of enhanced protein phosphatase 1 (PP1) and loss of phospholamban (PLN) phosphorylation. SERCA is modified and inhibited by PLN and hence inactivated causing calcium overload and subsequent heart failure.

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