

# Pressure Overload Promotes HMGB1 Signaling in the Ischemic-reperfused Heart

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## Abstract

Damage-Associated Molecular Patterns (DAMPs) are released following tissue injury and can activate pro-inflammatory pathways. Prototypical DAMPs include the High-Mobility Group Box 1 (HMGB1) protein which is released from necrotic cells. In turn, HMGB1 promotes inflammation via mechanisms likely involving phosphorylation/activation of c-Jun-NH2-Terminal Kinase (JNK) and production of the inflammasome-associated cytokine, interleukin-1 $\beta$  (IL-1 $\beta$ ). We have shown that pressure overload increases infarct size in association with poorer functional recovery of the heart subjected to an ischemia-reperfusion insult. This study tested the hypothesis that pressure overload augments HMGB1 expression in association with increased IL-1 $\beta$  generation and JNK phosphorylation thereby contributing to increased tissue injury. Accordingly, hearts of male Sprague-Dawley rats were subjected to an ischemia (40 min) reperfusion (15 min) insult with perfusion pressure set at either 80 or 160 cm H<sub>2</sub>O. Thereafter, hearts were processed for flow cytometry and Western blot studies. The ischemic-reperfused hearts subjected to the high pressure displayed (a) greater expression of HMGB1 and IL-1 $\beta$  which were shown to also be generated by cardiomyocytes, (b) increased phosphoJNK levels and c) increased necrotic and apoptotic cell death. Collectively, the results suggest that pressure-related upregulation of HMGB1 signaling contributes to the pro-inflammatory response of the ischemic-reperfused heart involving augmented JNK activation and IL-1 $\beta$  generation.

**Keywords:** Pressure; Heart; Ischemia-reperfusion; JNK; HMGB1; Interleukin; Cell death

## Introduction

Prompt reperfusion of ischemic myocardium is the most effective means of limiting tissue injury. Paradoxically, however, reperfusion of ischemic myocardium inflicts injury causing cell death via necrosis and apoptosis [1,2]. A critical event in this process is the release of Damage-Associated Molecular Patterns (DAMPs), with the nuclear protein High Mobility Group Box 1 (HMGB1) being recognized as a prototypical danger signal [3,4]. Indeed, HMGB1 plays a pivotal role in Ischemia-Reperfusion (IR) injury in the heart, brain and the liver as evidenced by its inhibition conferring protection [4-6].

The pathogenic mechanisms for HMGB1-induced tissue injury include its interaction with cryopyrin (Nalp3/Nlrp3), an intracellular Nod-like receptor, which functions as a danger signal sensor. Cryopyrin activation causes recruitment of apoptosis speck-like protein which contains a caspase-recruitment domain thereby resulting in the formation of the inflammasome. The latter is a multiprotein complex which is necessary for caspase-1 activation and subsequent release of the proinflammatory cytokine, interleukin-1 $\beta$  (IL-1 $\beta$ ) [7,8]. Another mechanism relates to HMGB1-mediated phosphorylation and activation of c-Jun NH2-Amino Terminal Kinase (JNK) which regulates inflammatory response following cardiac IR injury [9-14].

We have previously shown that pressure overload exacerbates oxidative/nitrosative stress and intracellular calcium overload associated with greater induction of the mitochondrial permeability transition pore, thereby exacerbating cell death/infarct size of the ischemic-reperfused heart [2]. Further, we have shown that pressure overload exacerbates endogenous cardiac inflammatory response to an IR insult [15,16]. Thus, the present study tested the hypothesis that pressure overload is a critical determinant of HMGB1 generation which, in turn, exacerbates IL-1 $\beta$  generation and augments phosphorylation/activation of JNK thereby contributing to inflammation and greater cell death in the ischemic-reperfused heart.

## Methods

Male Sprague-Dawley rats (9-11 weeks of age; Harlan Laboratories, Indianapolis, IN, USA) were obtained and housed in a room maintained at constant humidity (60  $\pm$  5%), temperature (24  $\pm$  1°C) and light cycle (0600-1800 h). The use of animals for this study was approved by the GRU institutional animal care and use committee.

For isolated heart perfusion experiments, animals were heparinized (1000 U/kg) and decapitated prior to removing the hearts and perfusing them on a Langendorff apparatus [15-17]. The perfusion medium was standard Krebs-Henseleit buffer (37°C) containing 11 mM glucose and equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub>; the perfusion pressure was set at either 80 or 160 cm H<sub>2</sub>O (i.e., 59 or 118 mm Hg, respectively). It is noteworthy that studies utilizing the isolated heart preparation typically use pressure heads of 100-120 cm H<sub>2</sub>O. In pilot studies, we initially established that pressures of 80 and 160 cm H<sub>2</sub>O provide the opportunity to examine pressure-related mechanisms that regulate cell fate. Indeed, these perfusion pressures have allowed us to readily decipher worsening or amelioration of cell death/infarct size by pharmacological agents or interventional modalities of post-conditioning as well as pressure loading and unloading [2,15-17]. Following a period of stabilization (25 min), each heart was subjected to 40 min of global ischemia followed by 15 min of reperfusion.

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Thereafter, cardiac tissue was filtered through a cell strainer (BD Biosciences, Bedford, MA, USA) and centrifuged (1500 rpm, 10 minutes) to obtain single cell suspension for flow-cytometry based assays [15,16]. Additional hearts were freeze-clamped for subsequent assessment of phosphorylation status of JNK [16,17].

### Analytical flow cytometry

Commercially available antibodies against each protein of interest were used coupled with the use of a *FACSCalibur* flow cytometer (BD BioSciences, San Diego, CA, USA) as described previously [15]. Brain natriuretic peptide, a marker of cardiomyocytes, was used to determine whether these cells are a source of HMGB1 and/or IL-1 $\beta$ .

### Assessment of cell death

Assessment of necrosis and apoptosis was achieved using the flow-cytometry-based annexin V/7-Amino-Actinomycin D (7AAD) protocol [15]. Annexin V is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis; 7AAD is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Staining was performed according to the manufacturer's instructions (BD Biosciences, Bedford, MA, USA). In brief, cells were washed twice with cold PBS and then resuspended in Binding Buffer and gentle vortex prior to incubation with annexin V antibody and 7-AAD. Cells were analyzed by flow cytometry within 1 hour after adding binding buffer.

### Western blotting

Total JNK and phosphoJNK levels of hearts were determined as described previously;  $\beta$ -actin was used to confirm equal protein loading [16,17].

### Antibodies and kits

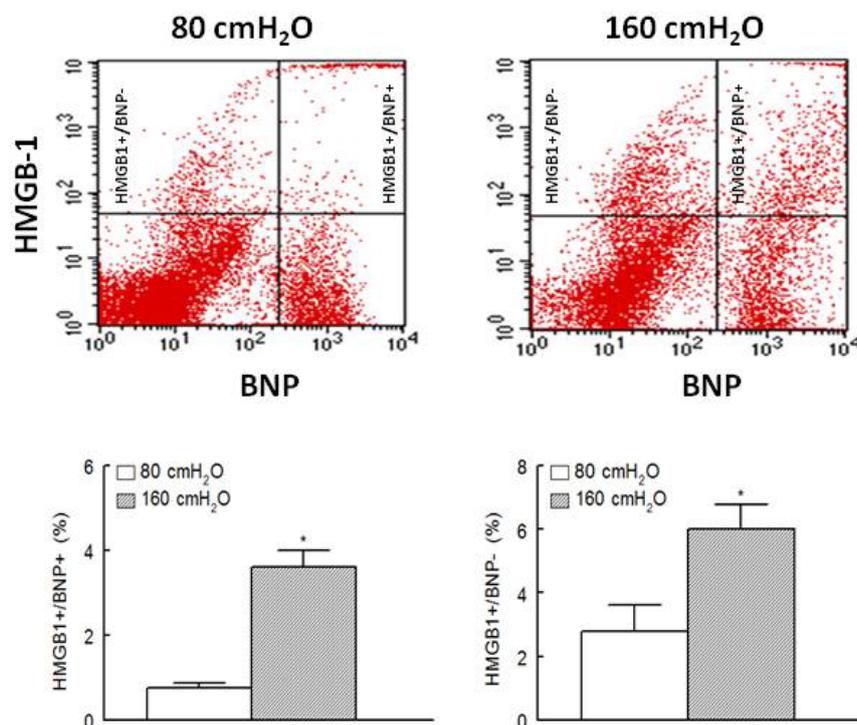
The primary, rabbit polyclonal, antibodies against IL-1 $\beta$  (biotin conjugated), and BNP were purchased from abcam (Cambridge, MA, USA) while that against HMGB1 was obtained from IBL Intl Corp (Toronto, Canada). Rabbit monoclonal antibodies against JNK and phosphoJNK (Thr 183/Tyr185) were purchased from Cell Signaling (Danvers, MA, USA) while rabbit anti-actin was obtained from Sigma Aldrich (St. Louis, MO, USA). Apoptosis Detection Kit was obtained from BD Biosciences (Bedford, MA, USA) and used following the manufacturer's instruction.

### Statistics

Data were analyzed using the Student's t-test to establish significance ( $p < 0.05$ ) between groups. Data are reported as means  $\pm$  SEM.

### Results

In order to determine whether cardiomyocytes are cellular sources of HMGB1 and/or IL-1 $\beta$ , assessment of their expression was carried out using BNP as a cardiomyocyte marker. As shown in Figure 1, although cardiomyocytes generate HMGB1 (i.e., HMGB1+/BNP+), non-cardiomyocytes seemingly are the major source of HMGB1 (i.e., HMGB1+/BNP-) in ischemic-reperfused hearts as revealed by a 3.7 fold higher percentage of non-cardiomyocytes than cardiomyocytes which were positive for HMGB1 at low pressure. Importantly, hearts perfused at high pressure displayed a marked increase in HMGB1+ cells compared to those perfused at low pressure. However, the relative increase in HMGB1+ cells was greater for BNP+ than BNP- cells (i.e., about 4.7 fold vs. 2.1 fold for high vs. low pressure) suggesting that



**Figure 1:** Panels show representative dot matrices for cardiac cell preparations from ischemic-reperfused hearts, subjected to either 80 or 160 cm H<sub>2</sub>O, which were positive for HMGB1, with or without reactivity for the brain natriuretic peptide (BNP; a marker of cardiomyocytes). On the other hand, bar graph shows means  $\pm$  SEM (%) of HMGB1+/BNP+ and HMGB1+/BNP- cells (n=5 hearts/group). \*  $p < 0.05$  compared to the other group.

pressure overload causes greater augmentation of HMGB1 expression in cardiomyocytes than other cardiac cells in ischemic-reperfused hearts. Figure 2 shows a similar pattern for IL-1 $\beta$  as revealed by expression of IL-1 $\beta$  in both cardiomyocytes and non-cardiomyocytes with the effect more prominent for the latter (e.g., about 9 fold greater for non-cardiomyocytes than cardiomyocytes at low pressure). Further, the relative increase in IL-1 $\beta$ + cells was greater for BNP+ than BNP- cells in hearts subjected to high, than low, pressure (i.e., about 13 fold vs. 4 fold for high vs. low pressure) suggesting pressure-related differential regulation of cardiomyocyte IL-1 $\beta$  generation in ischemic-reperfused hearts.

Figure 3 shows representative blots for total JNK1/2, phosphoJNK1/2 (pJNK1/2) and  $\beta$ -actin for ischemic-reperfused hearts subjected to the low or high pressure. Also shown are the ratios of pJNK1/2 to total JNK1/2 expressed as the percent of hearts subjected to low pressure. The results indicate significant increase in phosphorylation of JNK1/2 in hearts subjected to high, compared to low, pressure.

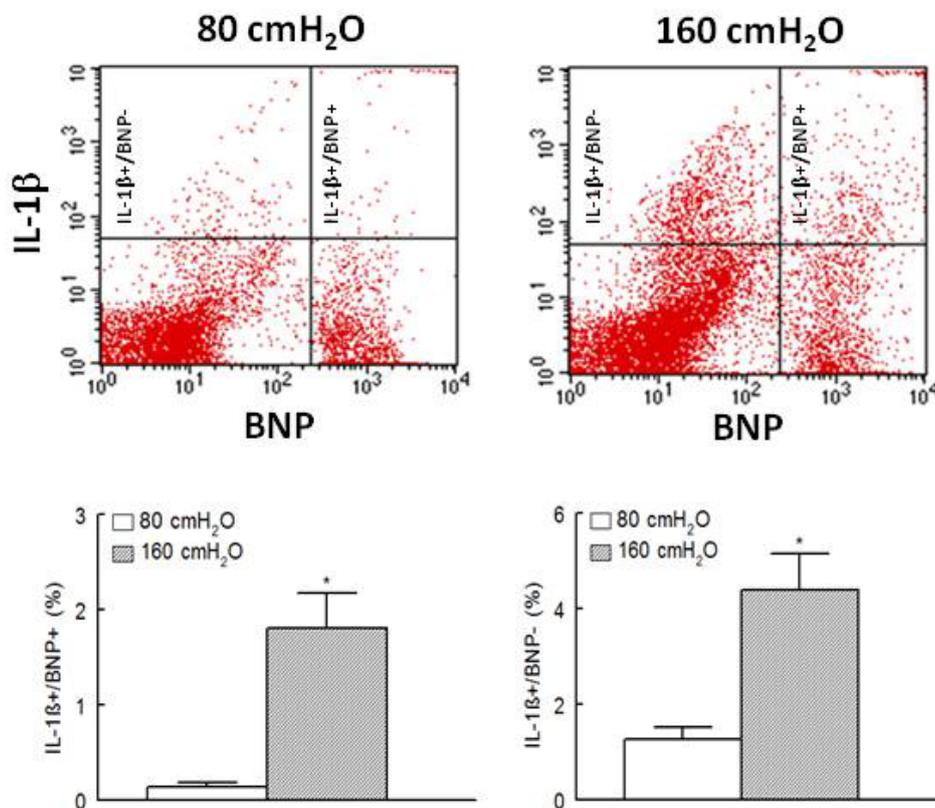
Figure 4 shows representative scatter plots for necrotic (7AAD+), necrotic/apoptotic (7AAD+/annexin V+) and early apoptotic (annexin V+) cells as well as percent of each type of cell death/damage in ischemic-reperfused hearts subjected to low and high pressure. Pressure overload resulted in significant increases in each type of damaged/dead cells in ischemic-reperfused hearts. As a result, the percent of undamaged/normal cells were significantly lower in hearts subjected to high than low pressure (Figure 4). It is noteworthy that time-controlled normoxic control hearts, perfused at either 80 or 160 cm H<sub>2</sub>O, display a very small

percentage of total damaged/dead cells (i.e., about 2 to 6%, respectively) and that the vast majority of cells (i.e., about 94-98%) prepared from these hearts are comprised of undamaged/normal cells [15,16].

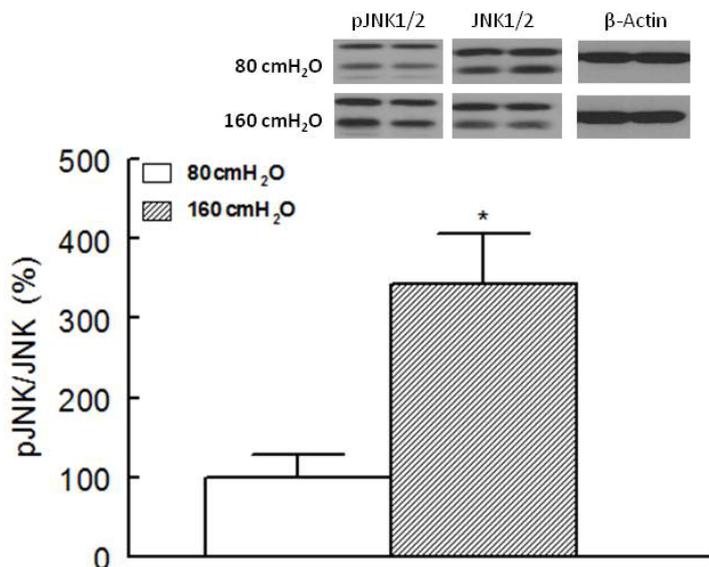
## Discussion

The present study shows that pressure/mechanical stress is a key determinant of HMGB1 and IL-1 $\beta$  expression as well as phosphorylation of JNK in association with marked increased in necrosis and apoptosis in the ischemic reperfed heart. Importantly, cardiomyocytes are a major source of pressure-related upregulation of HMGB1 and IL-1 $\beta$  in response to an IR insult. Collectively, these observations corroborate our recent studies indicating that myocardial load/mechanical stress is a pivotal determinant of regulation of the endogenous cardiac inflammatory mechanisms [15,16].

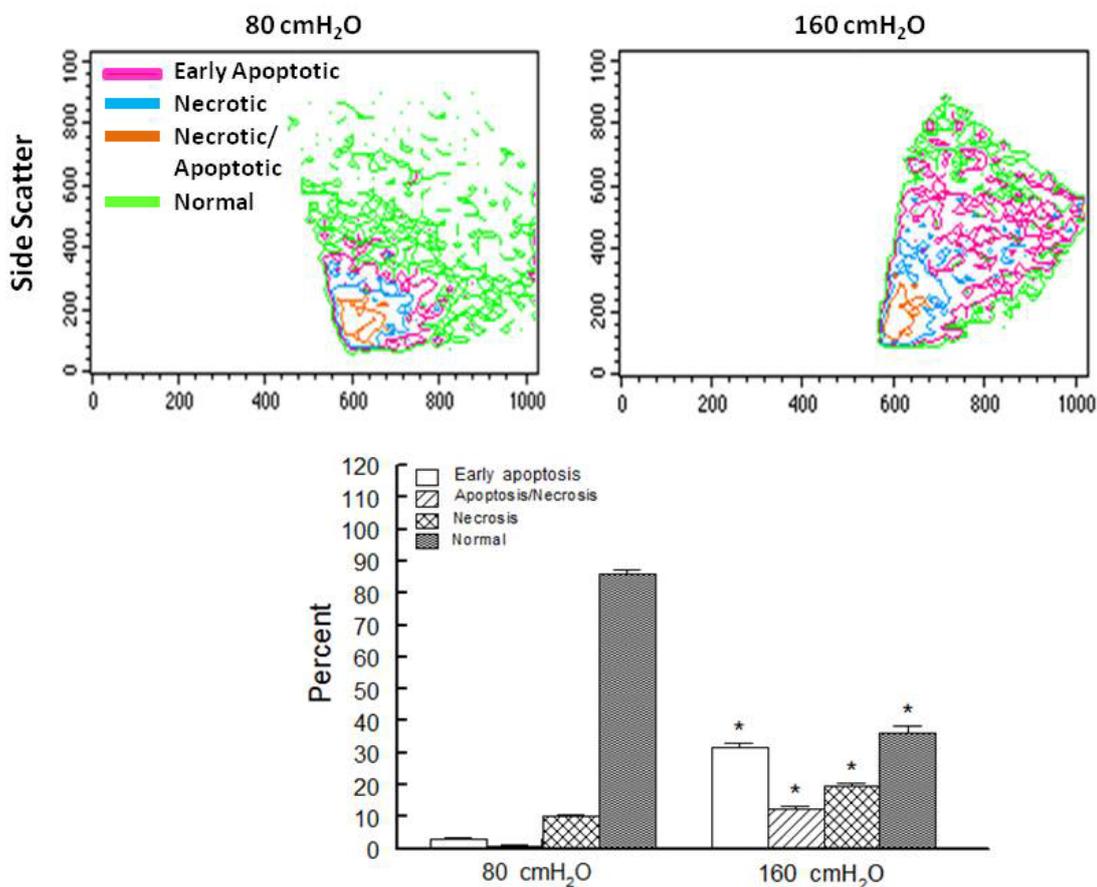
HMGB1 is functionally recognized as an “alarmin” or “danger signal”. It can be released from cells to inform adjacent or remote cells of prevailing infection and/or tissue injury. Consequently, appropriate immune responses are mounted to ultimately limit injury and protect the affected tissue. A number of immune cells such as neutrophils, macrophages and monocytes can secrete HMGB1 [3,18]. On the other hand, HMGB1 release from non-immune cells was initially attributed to passive release from necrotic cells. Indeed, HMGB1 release occurs following in vitro anoxia/reoxygenation insult to cardiomyocytes and in response to an in vivo IR insult to the heart [19]. Further, treatment with an HMGB1 antagonist, rAbox, significantly reduces infarct size and markers of cardiac tissue injury [19]. These observations indicate that release of HMGB1 from necrotic cells exerts deleterious effects.



**Figure 2:** Panels show representative dot matrices for cardiac cell preparations from ischemic-reperfused hearts, subjected to either 80 or 160 cm H<sub>2</sub>O, which were positive for IL-1 $\beta$ , with or without reactivity for the brain natriuretic peptide (BNP; a marker of cardiomyocytes). On the other hand, bar graph shows means  $\pm$  SEM (%) of IL-1 $\beta$ + /BNP+ and IL-1 $\beta$ + /BNP- cells (n=5 hearts/group). \*p<0.05 compared to the other group.



**Figure 3:** Effect of perfusion pressure on phosphorylation status of JNK1/2. Hearts were perfused at either 80 or 160 cm H<sub>2</sub>O and subjected to an ischemia (40 min) reperfusion (15 min) insult followed by freeze clamping for subsequent Western blotting. Densitometry data were initially expressed as the ratio of pJNK1/2 to total JNK1/2 for hearts perfused at each pressure. Thereafter, the pJNK/JNK ratio was expressed as percent of hearts subjected to the low pressure; data are means ± SEM of n=6 hearts/group. Also shown are representative blots for pJNK1/2, JNK1/2 and β-actin for each group. \*p<0.05 compared to the other group.



**Figure 4:** Panels show representative scatter plots for necrotic, necrotic/apoptotic and early apoptotic cells in cardiac cell preparations from ischemic-reperfused hearts subjected to 80 or 160 cm H<sub>2</sub>O. Bar graphs show means ± SEM of percent of damaged/dead or normal cells for each group. \* p<0.05 compared to the 80 cm H<sub>2</sub>O group.

Importantly, however, recent studies show that HMGB1 can also be produced and secreted by viable cardiomyocytes (e.g., in response to lipopolysaccharide) in a highly regulated process involving toll-like receptor 4 and phosphatidylinositol 3-kinase  $\gamma$  [3]. Consistent with these studies, we now show that pressure/mechanical stress is also a critical determinant of myocardial HMGB1 generation. Indeed, gating on viable cells in our flow-cytometry approach lends further credence to the notion that viable cardiac cells can upregulate HMGB1 generation. Importantly, using BNP as a marker of cardiomyocytes, we also showed that increased perfusion pressure upregulates HMGB1 expression in cardiomyocytes. Collectively, these observations substantiate the notion that myocardial load/mechanical stress is a critical determinant of the alarmin response in the ischemic-reperfused heart. Nonetheless, it is noteworthy, that while endogenous HMGB1 generation is linked to myocardial ischemia reperfusion injury, the impact of exogenous HMGB1 on the heart is less clear with some authors suggesting a cardioprotective effect while others indicating worsening of cardiac IR injury [4,12,19,20].

Multiple mechanisms contribute to HMGB1-mediated tissue injury. A prominent mechanism relates to inflammasome formation, caspase 1 activation and generation of the pro-inflammatory cytokine, IL-1 $\beta$  [21]. Indeed, cardiac fibroblast upregulation of inflammasome, and consequent IL-1 $\beta$  generation, is an important mediator of cardiac IR injury [22,23]. It is noteworthy that while non-cardiomyocytes (i.e. BNP- cells) expressed HMGB1 and IL-1 $\beta$  in this study, pressure-related augmented expressions of HMGB1 and IL-1 $\beta$  were more marked for cardiomyocytes suggesting that myocardial load differentially impacts their generation. Nonetheless, the generation of IL-1 $\beta$  in this study is consistent with our previous demonstration of production of other pro-inflammatory cytokines including IL-17 and IL-23 in ischemic-reperfused hearts and their augmentation by pressure/mechanical stress [2,15,16]. It is noteworthy that, once generated, IL-1 $\beta$  can form complexes with HMGB1 to further boost pro-inflammatory cytokine production; this mechanism is suggested to play a pathogenic role in arthritic joint disease. Accordingly, it is shown that HMGB1/IL-1 $\beta$  complexes increase expression of microsomal prostaglandin E synthase-1 and cyclooxygenase 2 in synovial fibroblasts suggestive of perpetuation of inflammatory and destructive activities in rheumatoid arthritis [24,25]. Whether complex formation of HMGB1 with other cytokines (e.g., IL-1 $\beta$ , IL-17) contributes to cardiac IR injury and its exacerbation by pressure overload remains to be established.

Several lines of evidence support an important role for JNK in mediating the effects of HMGB1 including a) regulation of autophagy in human myeloid leukemia cells, b) proliferation, migration and fibrotic effects of hepatic stellate cells, c) inflammatory response of endothelial cells and d) inflammatory response of human bronchial epithelial cells [9-11,13]. Importantly, the cardioprotective effect of glycyrrhizin is exerted through blockade of HMGB1-dependent phosphoJNK/Bax pathway [12]. We now show that the pressure-related exacerbation of HMGB1 expression is associated with significant increase in phosphoJNK levels in ischemic-reperfused hearts. These observations suggest involvement of HMGB1/JNK pathway in upregulation of endogenous cardiac pro-inflammatory mechanisms. In this context, it is noteworthy that JNK is a signaling protein of Endoplasmic Reticulum (ER) stress response and its phosphorylation/activation is consistent with pressure-related increased expression of the classical marker of ER stress response protein, the growth-arrest and DNA-damage associated protein 153; ER stress response is an important regulator of inflammation and cell death [9,15-16].

Pressure overload was associated with marked increase in necrotic and apoptotic cell death in ischemic-reperfused hearts. It is plausible that increased HMGB1 expression and associated upregulation of IL-1 $\beta$  and phosphoJNK contribute importantly to perpetuation of cardiac inflammation and greater susceptibility of pressure-overloaded heart to IR injury. Indeed, the link between HMGB1 and tissue injury is well-established in light of a number of reports demonstrating that inhibition of HMGB1 confers protection in several conditions including cardiac IR injury [3-5,12]. Further, HMGB1 serum levels are correlated with infarct size and residual ejection fraction in patients with ST elevation and non-ST elevation [26].

In conclusion, pressure/mechanical stress is a major determinant of HMGB1 expression, IL-1 $\beta$  generation and JNK activation in the ischemic-reperfused heart. These observations are of relevance for systemic hypertension, a very prevalent disorder which is common in patients with coronary artery disease [2]. Further, systemic hypertension represents years of pressure overload on the myocardium. Thus, further investigation of pressure-related molecular mechanisms which contribute to endogenous cardiac inflammation and determine the fate of the cardiomyocyte is warranted.

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