

BRD2 protects the rat H9C2 cardiomyocytes from hypoxia-reoxygenation injury by targeting Nrf2/HO-1 signaling pathway

YINGCUN LIU^{1*}, YUQING FU^{2*}, XIN XUE¹, GANG TANG³ and LIANGYI SI¹

¹Department of Cardiology, The Third Affiliated Hospital, Chongqing Medical University, Chongqing 401120; ²Department of Cardiology, The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, Guangdong 518000; ³Department of Cardiovascular Medicine, Shapingba Hospital Affiliated to Chongqing University, Chongqing 400030, P.R. China

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Abstract. Myocardial ischemia-reperfusion (I/R) injury is a common complication of acute myocardial infarction following percutaneous coronary intervention, but there are currently no effective pharmacological targets for adjuvant therapy due to a lack of knowledge of I/R injury mechanisms in cardiomyocytes. To evaluate the effects of hypoxia-reoxygenation on the plasma proteome of cardiomyocytes and prospective therapeutic targets, five sets of H9C2 cardiomyocytes from rats were cultured under various hypoxic circumstances. Using Cell Counting Kit-8 (CCK8) and lactose dehydrogenase (LDH) release assays, the cell viability and LDH release of H9C2 cells were analyzed. Proteome sequencing was then performed on cardiomyocytes to show the quantitative protein changes during the I/R injury process. After hypoxia/reoxygenation, bromodomain-containing protein 2 (BRD2) expression was evaluated. After administering the BRD2 inhibitor dBET1, the expression of nuclear factor erythroid 2-related factor 2/haem oxygenase-1 (Nrf2/HO-1) was identified. The results showed that in the group exposed to 4 h of hypoxia followed by 4 h of reoxygenation (H/R4), the cell survival rate was

dramatically reduced, although the apoptotic rate and LDH were much higher than in the normal oxygen group. In addition, the expressions of 2,325 proteins differed considerably between these two groups, with 128 upregulated and 122 downregulated proteins being discovered in the H/R4 group. After 4 h of reoxygenation, the BRD2 expression was increased. Following the addition of dBET1 to suppress BRD2, the expression of Nrf2/HO-1 was reduced, but the rate of apoptosis increased. In conclusion, through the Nrf2/HO-1 signaling pathway, BRD2 protects cardiomyocytes from damage caused by hypoxia/reoxygenation. This may have implications for novel treatment targets to minimize I/R damage to the myocardium.

Introduction

Acute myocardial infarction (AMI) is one of the deadliest diseases in the world (1,2). Although the extensive application of percutaneous coronary intervention (PCI) has improved the prognosis of AMI in recent years, abrupt reperfusion in the ischemic myocardium can lead to ischemia/reperfusion (I/R) injury in cardiomyocytes (3,4). However, the unclear mechanism of I/R injury limits the development of therapeutic strategies.

Studies have shown that the bromodomain (BRD)-containing protein family, including BRD2, BRD3, BRD4, BRD7 and BRD9, and the bromodomain, testis-specific (BRDT) protein (5), is associated with a variety of cardiovascular diseases. For instance, the upregulation of citrate cycle genes by BRD2 may facilitate cardiac hypertrophy (6). BRD4 is involved in myocardial infarction (7), cardiac hypertrophy (8) and even cardiac remodeling and functions (9,10). In addition, the myocardial infarction-induced myocardial injury is regulated by BRD7 through activating Wnt/ β -catenin signaling (11). However, few studies have reported the role of the BRD family in myocardial I/R injury.

Thus, the objective of the present study was to investigate the role of the BRD family in myocardial I/R injury. Given that myocardial I/R injury is a condition involving a complex interplay of molecular mechanisms, such as intracellular calcium

Correspondence to: Professor Liangyi Si, Department of Cardiovascular Centre, The Third Affiliated Hospital, Chongqing Medical University, 1 Shuanghu Branch Road, Huixing Street, Yubei, Chongqing 401120, P.R. China
E-mail: sly@hospital.cqmu.edu.cn

Dr Gang Tang, Department of Cardiovascular Medicine, Shapingba Hospital Affiliated to Chongqing University, 44 Xiaolongkan New Street, Shapingba, Chongqing 400030, P.R. China
E-mail: tanggang2002@163.com

*Contributed equally

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overload, oxidative stress, disturbance of energy metabolism, apoptosis, vascular endothelial injury and neutrophil infiltration (12-15), proteomics was performed to investigate the changes of all proteins in a certain type of cell at the protein level and reveal the biological function of BRDs more accurately than transcriptome analysis (16). The present study also used the tandem mass tag (TMT) labelling quantitative technique combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to demonstrate the changes of all plasma proteins in myocardial cells during I/R injury. It is hoped that the present study could provide potential therapeutic targets by determining a possible relationship between the expression of BRDs and myocardial I/R injury. Understanding the relationship between BRD expression and cardiac damage can pave the way for the development of targeted interventions to protect cardiomyocytes following AMI. These insights may lead to novel treatment strategies aimed at improving patient outcomes in AMI.

Materials and methods

Cell lines and culture. Shanghai Cell Bank provided H9C2 cardiomyocytes with the serial number GNR 5. Cells were grown in At 37°C with 5% CO₂ in DMEM augmented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 10% streptomycin, and 1% penicillin (Thermo Fisher Scientific, Inc.).

Establishment of the cardiomyocyte hypoxia/reoxygenation model and experimental grouping. A hypoxia/reoxygenation (H/R) model was created by filling a Billups-Rothenberg hypoxic/anaerobic incubator with a mixture of 95% N₂ and 5% CO₂ (Chongqing Ruike Gas Co. Ltd.). Prior to the cells were treated with hypoxia, they were cultured in a medium (2 ml of DMEM) devoid of sugar and serum for 3 h with 5% CO₂ at 37°C. The H/R and hypoxia groups were then transferred to an anaerobic incubator containing a hypoxia gas mixture. The control group was also subjected to standard incubation conditions consisting of 5% CO₂ and 37°C. The six-well plates were taken away from the hypoxia for 4 h, and their original medium was swapped out for the sugared medium. After that, the cells spent time in a 5% CO₂ incubator at 37°C for 2, 4, and 8 h to re-oxygenate. The abbreviation 'H4' was used to represent hypoxia for 4 h, and the abbreviations 'H/R2' 'H/R4' and 'H/R8' used to represent hypoxia for 4 h and then reoxygenation for 2, 4, and 8 h, respectively.

Detection of the cell survival rate by CCK8. After cells were adhered for a day, both groups were cultured normally in a 5% CO₂ cell incubator, but the experimental group also received H/R treatment as described above. Cells in each group were treated with CCK-8 reaction solution following hypoxia and H/R, and incubated at 37°C for 1 h, in accordance with the instructions provided in the CCK-8 test kit. The OD values were determined by analyzing the microplates at 450 nm.

LDH release from H9C2 cardiomyocytes was analyzed using LDH release assays. Following the establishment of the H/R paradigm, the cell supernatants were collected, and LDH in cardiomyocytes was quantified as directed by the manufacturer

of the LDH kit (Nanjing Jian Cheng Bioengineering Institute; cat. no. A020-2).

Detection of apoptosis by flow cytometry. Following the establishment of a suitable H/R model, the cell cycle analysis was performed on a fluorescence-activated cell sorting flow cytometer (BD FACSCalibur; BD Biosciences). The cells were resuspended in DMEM. Annexin V FITC (20 µg/ml) and propidium (PI) (50 µg/ml) in 100 µl Annexin V binding buffer were added to the cells and incubated for 15 min at 4°C in the dark. The flow cytometer was used to analyze the samples for apoptosis. FlowJo v10.0 (FlowJo LLC) and fluorescence-activated cell sorting were used to analyze the data and calculate the proportion of apoptotic cells. The total apoptotic rate was calculated as the early apoptotic rate plus the late apoptotic rate.

Tandem mass tag (TMT) isobaric labelling. After the cardiomyocytes were washed with PBS, they were lysed using RIPA buffer. The protein concentration was measured by a BCA protein assay kit. Filter-aided sample preparation was used to digest 100 µg of proteins. The control group, hypoxia for 4 h and reoxygenation for 2, 4, and 8 h groups were labelled with 126, 127, 128, 129, and 130 Th, respectively, and the reoxygenation for 4 h group was labelled again with 131 Th. The samples were separated into eight fractions by pH12 reversed-phase chromatography. An Easy-nanoLC1000 liquid chromatograph and an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Inc.) were used for proteomic analyses. The Swiss-Prot database's differential proteins (Homo sapiens, release 2020_06; www.uniprot.org) were compared and screened using Protein Discovery Software 1.4 (Thermo Fisher Scientific, Inc.).

The Z-test was utilized to identify proteins with statistically significant changes in abundance (P<0.05) and then the significance of the hit was used to filter out proteins with 1.2-fold changes. Proteomics findings were uploaded to STRING (<https://string-db.org/>). Kyoto Encyclopedia of Genes and Genomes (KEGG) 2.4.1 (www.genome.jp/kegg/) was used to discover protein pathways. The identified proteins were evaluated using Gene Ontology (GO) terms in Metascape (<https://metascape.org/>).

Analysis of the cell proteome. The Proteome Discoverer 21 (Thermo Fisher Scientific, Inc.) program transformed the proteome data, which was then loaded into Mascot 2.2.0. algorithm (Matrix Science Ltd.) for retrieval. After exporting the data, the expression matrix containing all genes and phenotypes of the samples was subjected to Gene Set Enrichment Analysis (GSEA) software (version 4.0.3, Broad Institute) for the analysis of GO enrichment. Additionally, for a functional-level analysis of the differentially expressed genes, the present study utilized the Metascape database (https://metascape.org), an online tool that facilitates the annotation, visualization, and integrated discovery of gene lists (17). Through this tool, it was possible to gain functional insights into the GO enrichment and KEGG pathway analysis for the differentially expressed genes.

Western blotting. After observing a significant decrease in cell viability, an increase in LDH release and higher

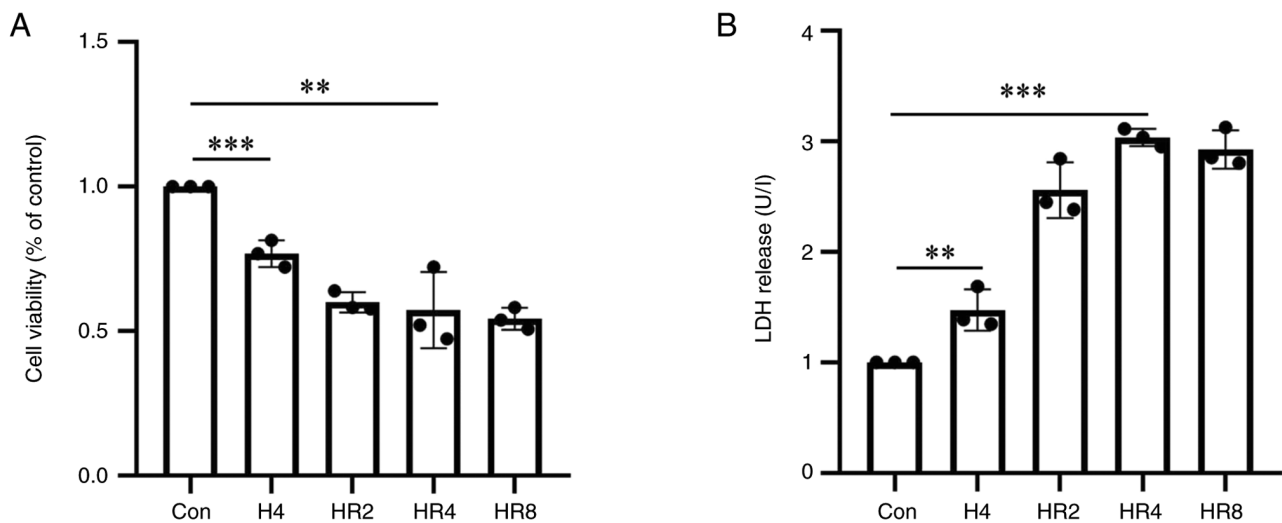


Figure 1. Assessment of cell viability and LDH release under hypoxia and reoxygenation conditions. Cell Counting Kit-8 and LDH assays were used to detect the changes in (A) the cell viability and (B) LDH release at different time points of hypoxia and reoxygenation, respectively. Statistical evaluation was performed using one-way ANOVA followed by Tukey's post hoc test. ** $P < 0.01$; *** $P < 0.001$. LDH, lactose dehydrogenase; H4, hypoxia for 4 h; H/R2, H/R4 and H/R8, hypoxia/reoxygenation for 2, 4 or 8 h.

apoptotic rates in the H/R4 group compared to the other groups, the present study mainly concentrated on examining the expression of BRD2 in the H/R4 group. Total protein of H/R4 group was recovered from isolated cardiomyocytes using standard methods and RIPA buffer. The protein concentration was determined with a BCA protein kit. Each lane contained 40 μg of protein that had been separated using 10% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (MilliporeSigma). The membranes were blocked for 2 h of room temperature with Tris-buffered saline containing 0.1% Tween 20, including 5% nonfat milk (TBST). Finally, the membranes were treated with anti-BRD2 (1:1,000; cat. no. 139690; Abcam) and anti-nuclear factor erythroid 2-related factor 2 (Nrf2) (1:1,000; cat. no. 13901; Cell Signaling Technology) and anti-Vinculin (1:1,000; cat. no. 16396-1-AP; Proteintech Group, Inc.) at 4°C overnight. Membranes were incubated with an HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody for 1 h at room temperature after the primary antibody incubation (1:500; Cell Signaling Technology). The detection was made using a Bio-Rad ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.).

Determination of the effective concentration of dBET1. dBET1 is a proteolysis-targeting chimera that can result in selective degradation of BET proteins (18). In order to delve deeper into the regulatory pathway indicated in the proteomics of BRD2, the expression of haem oxygenase-1 (HO-1) and Nrf2 before and after the administration of dBET1 was investigated. For the concentration-response experiment, 2×10^5 cells were cultured in 2 ml DMEM containing 20 nM dBET1 (Selleck Chemicals). The cells were cultivated in an incubator containing 5% CO_2 at 37°C for 48 h and oxygen-deprived for 4 h in a Billups-Rothenberg hypoxic/anaerobic incubator containing a mixture of gases (95% N_2 , 5% CO_2). Then, western blotting was used to determine the BRD2 protein expression. At a dose of 20 nM, the expression of BRD2 was markedly suppressed.

This concentration was used for the subsequent experiment. Furthermore, the cell viability, LDH release, and apoptotic rates in the H/R4 group after the administration of dBET1 were also detected, using the same previously mentioned methods.

Statistical analysis. All statistical analyses were performed with SPSS 21.0 (IBM Corp.). The figures indicate the mean and standard deviation of the mean (SD). Differences among three groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using GraphPad Prism software, as specified in the figure legends. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of the (H/R) model. The CCK-8 results revealed a reduction in cell survival as reoxygenation time increased (Fig. 1A). The difference in cell survival rates between the control group and the 4 h reoxygenation group was statistically significant ($P < 0.01$). The LDH results indicated that as reoxygenation time increased, cell damage was exacerbated. In terms of cell damage, the difference between the 4 h reoxygenation group and the control group was statistically significant ($P < 0.001$; Fig. 1B). Flow cytometry demonstrated that the apoptosis rate of the 4 h hypoxia group was significantly higher than that of the control group ($P < 0.01$; Fig. 2) and that the apoptotic rate rose as reoxygenation time increased. The difference in the apoptotic rate between the 4 h reoxygenation group and the control group was statistically significant ($P < 0.001$; Fig. 2). It was determined that 4 h of hypoxia and 4 h of reoxygenation were ideal for the development of cardiomyocytes.

Screening and global analysis of significantly different proteins in the H/R4 group. A total of 2,325 significantly differentially expressed proteins were identified by TMT

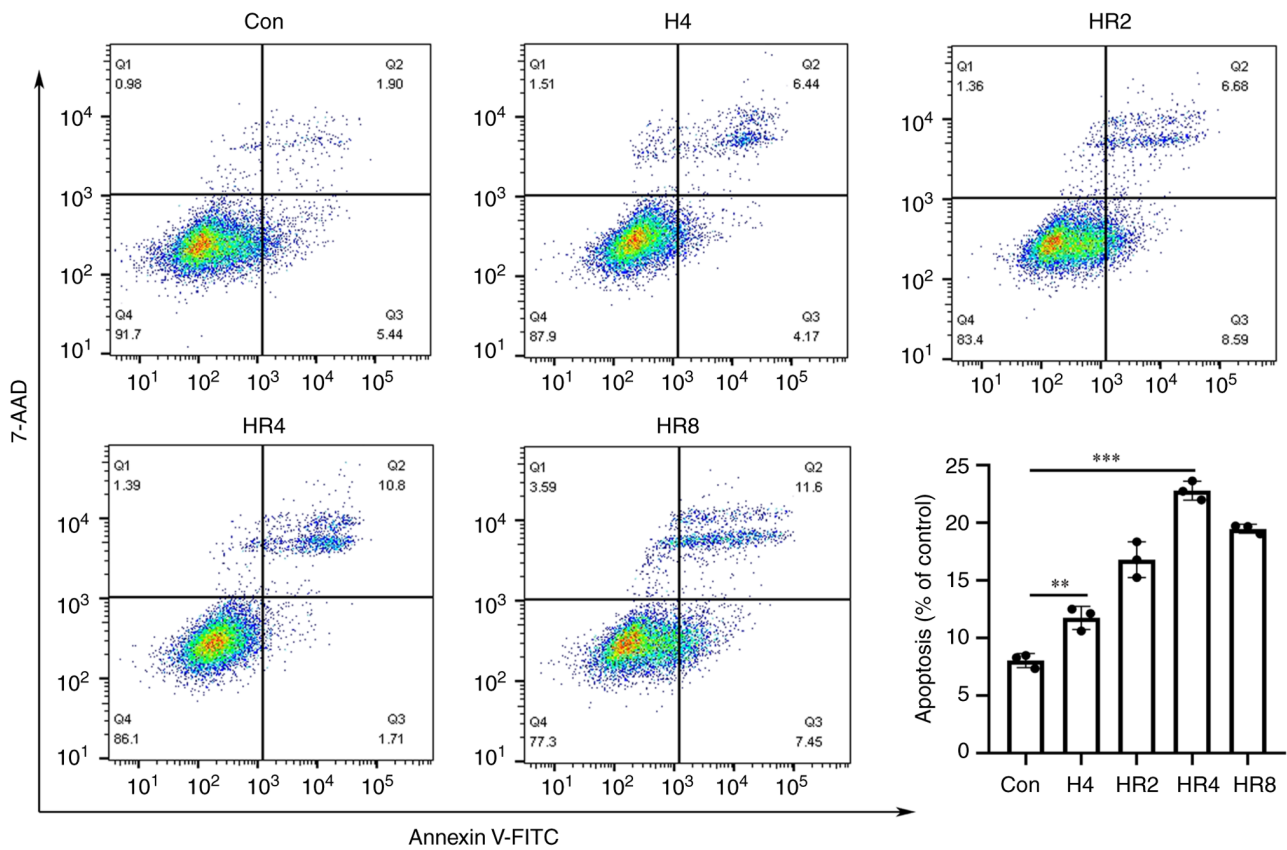


Figure 2. Changes in the apoptotic rate at different time points detected by flow cytometry. Statistical analysis was conducted by one-way ANOVA followed by post hoc Tukey's t test. ** $P < 0.01$; *** $P < 0.001$. Con, control; H4, hypoxia for 4 h. H/R2, H/R4 and H/R8, hypoxia/reoxygenation for 2, 4 or 8 h.

labelling combined with reverse-phase liquid chromatography (RP-HPLC). Using a bioinformatics method, the proteins of each experimental group were screened according to the standard of fold change >1.2 or <0.8 and P-value (t-test) <0.05 . As shown in Fig. 3A, compared with the normal control group, there were 267 significantly different proteins under hypoxia for 4 h, 286 significantly different proteins under H/R2, 250 significantly different proteins under H/R4, and 247 significantly different proteins under H/R8. Among them, 119 proteins were involved in the process of hypoxia and reoxygenation. A total of 49 proteins appeared in H/R4 but were not involved in the process of simple hypoxia for 4 h.

Analysis of the effects of the significant differentially expressed proteins in the H/R4 group. The present study identified 250 significantly differentially expressed proteins (Fig. 3B and C) in the H/R4 group concerning the GO and KEGG databases. The GO analysis results were as follows: Among biological processes, the differentially expressed protein functions were involved in regulating nucleic acid metabolism, vascular development, lipid metabolism and metal ion transport among others. Among molecular functions, the protein functions were mainly focused on regulating enzyme molecular functions. In the cellular component category, these differentially expressed proteins were mainly distributed in the cytoplasm, endometrium system, and extracellular gap. KEGG analysis showed that differentially expressed proteins resulting from myocardial injury induced by H/R were correlated with tight

junctions, protein degradation pathways and signaling pathways among others.

Cluster analysis of differentially expressed proteins in the H/R4 group. The protein cluster analysis findings (Fig. 4A and B) revealed that the histone family proteins H2AFZ, H2AFJ and H2AFY increased during hypoxia and reduced following reoxygenation. Histone H3 (H3F3B) expression also rose during hypoxia and dropped following reoxygenation, while the expression levels of Ptsg2, Serprine1 and Junb proteins fell during hypoxia for 4 h and increased following reoxygenation for 4 h. In the heat map analysis, which resembled the volcano plot of the protein screening, protein clustering was evident. The protein interaction network map (Fig. 4C) revealed that in H/R4, the H3F3B expression was dramatically reduced and the BRD2 expression was significantly enhanced compared to the control group. The increased protein Gyr was consistent with the volcano plot.

Overexpression of BRD2 regulated by Nrf2/HO-1 signaling pathway in H/R4 group. Western blot analysis revealed that the expression of BRD2 was dramatically increased after 4 h of exposure to hypoxia and reoxygenation, corroborating the protein cluster analysis. In addition, when the BRD protein inhibitor dBET1 was added, the expression of BRD2 fell dramatically ($P < 0.01$; Fig. 5A). This H/R mechanism was related to the Nrf2/HO-1 signaling pathway, and dBET1 reversed the upregulation (Fig. 5B).

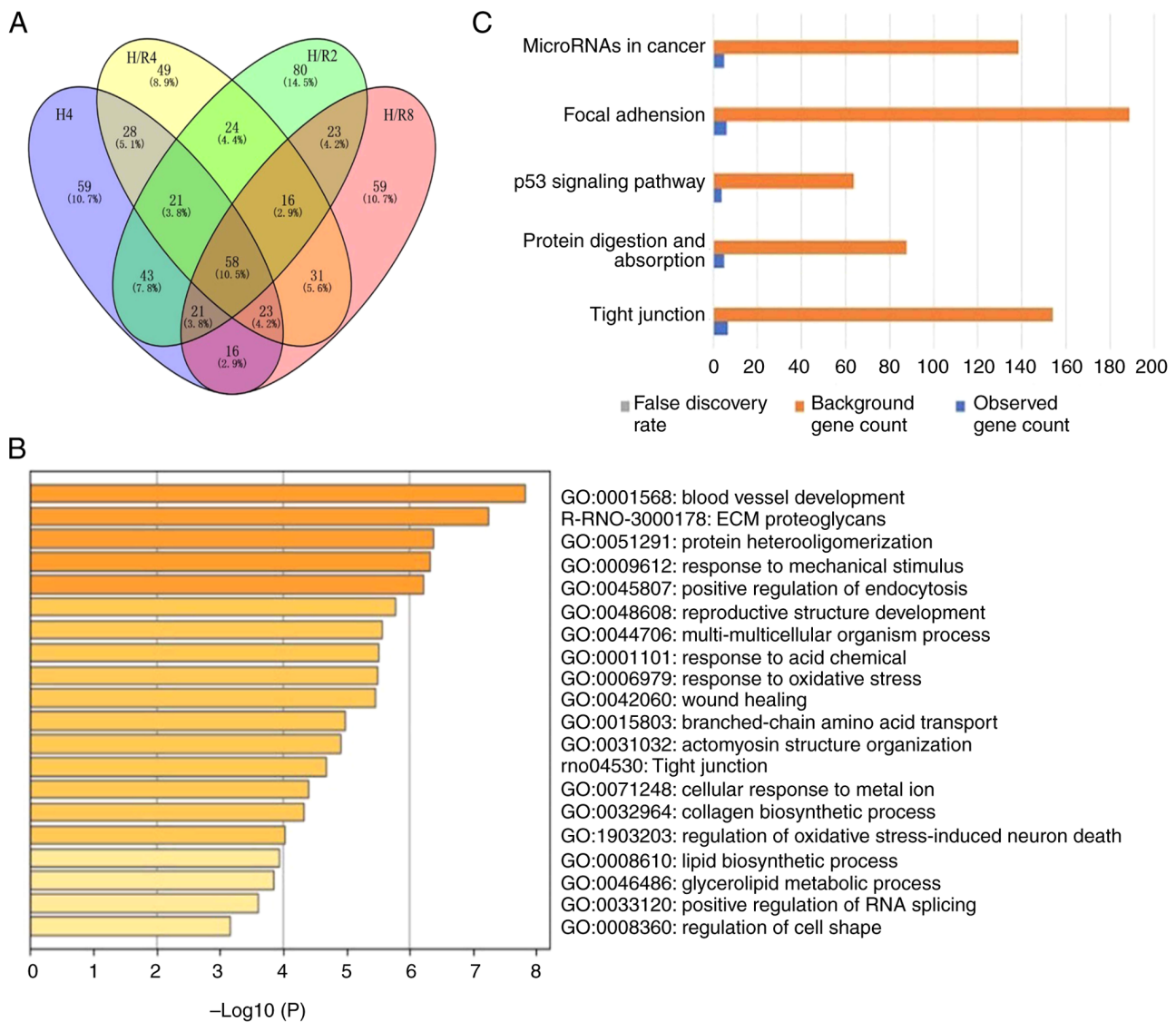


Figure 3. Analysis of differentially expressed proteins and pathway enrichment under hypoxia/reoxygenation conditions. (A) Venn diagram shows the number of main differentially expressed proteins in each group. (B) GO analysis and (C) KEGG signaling pathway analysis of significantly differentially expressed proteins in H/R4. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; H/R4, hypoxia/reoxygenation for 4 h.

Effects of BRD2 protein inhibition on cardiomyocyte's apoptosis, cell viability, and release of LDH. Flow cytometry analysis revealed that, relative to the control group, cardiomyocyte apoptosis was elevated in the H/R4 group and in the H/R4 group following administration of the BRD2 inhibitor dBET1 ($P < 0.05$; Fig. 6A). Using the CCK-8 assay, it was also determined that the addition of dBET1 significantly lowered cell viability ($P < 0.01$; Fig. 6B). After addition of dBET1, the release of LDH was enhanced ($P < 0.001$; Fig. 6C).

Discussion

To the best of the authors' knowledge, this is the first study to demonstrate that BRD2 can protect cardiomyocytes from H/R-induced damage by targeting the Nrf2/HO-1 signaling pathway. In addition, the present study enhanced the existing knowledge of the mechanism of myocardial I/R damage from the viewpoint of proteomics and revealed the relevance of BRD2 as a possible therapeutic target.

Multiple signaling pathways have been linked to the process of myocardial I/R damage (19-22), but there are still no suitable interventions to alleviate I/R injury during cardiac surgery due to the poor understanding of proteome changes caused by the lack of high-throughput protein detection technology. Given that the pathophysiological process of H/R damage in cardiomyocytes is comparable with that of myocardial I/R injury, a cardiomyocyte H/R model was developed to replicate the myocardial I/R process. TMT labeling in conjunction with HPLC was used to identify alterations in the proteome of H9C2 cardiomyocytes and a total of 2325 proteins with differential expression were discovered. Through a proteomic analysis, Li *et al* (19) demonstrated that when human cardiac microvascular endothelial cells were subjected to I/R injuries, the expression of 30 proteins changed, highlighting their potential roles in cell proliferation, stress response, and regulation of metabolic process upon I/R injury. The present study also conducted in-depth information mining of proteins related to intimal system changes with the development of

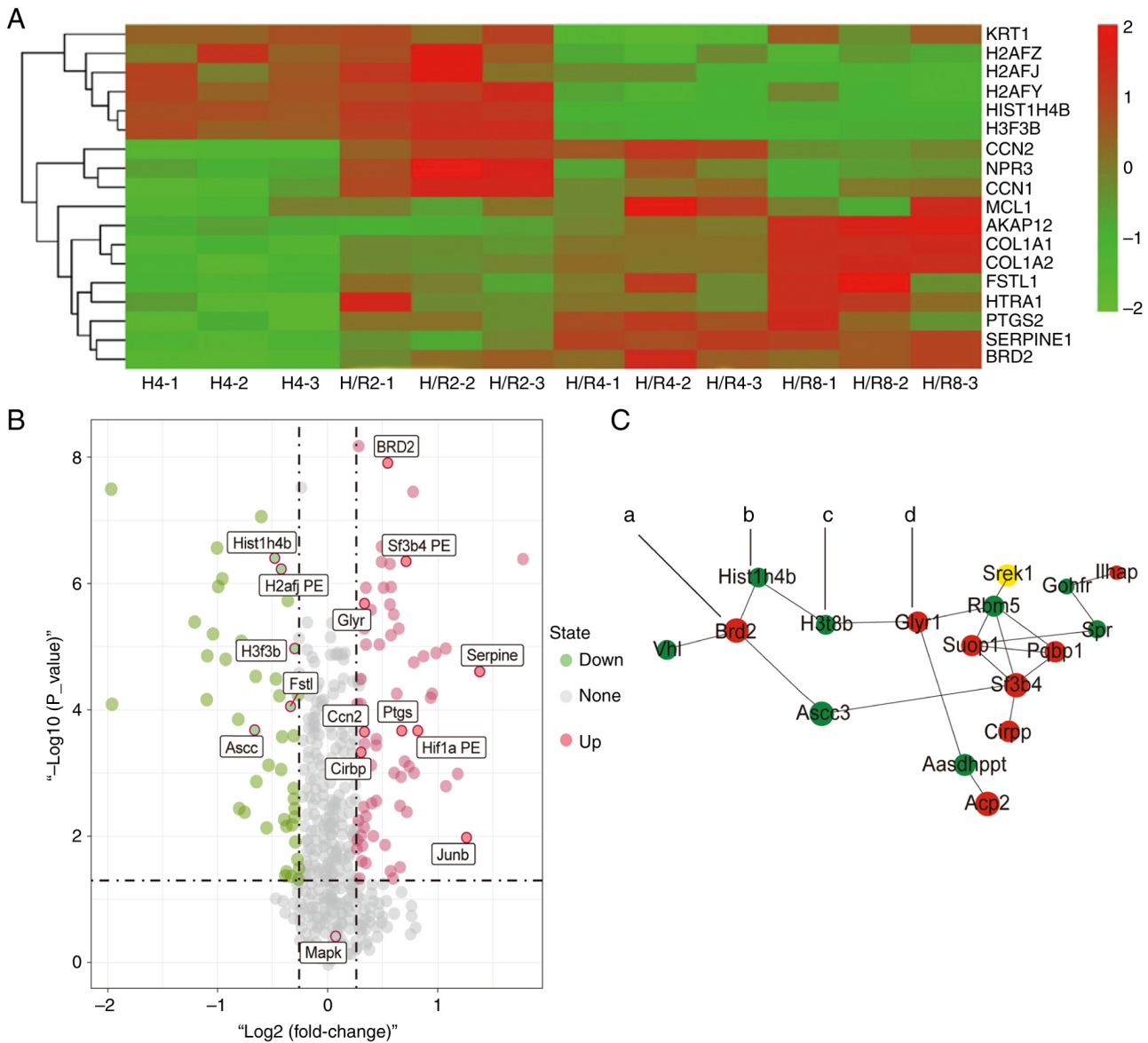


Figure 4. Cluster analysis of core differentially expressed proteins in hypoxia-reoxygenation for 4 h by heat map and volcano plot. (A) Heat map analysis of the TMT abundance data for 18 representative proteins identified in the whole proteome. (B) Volcano plot of differentially expressed proteins in each group. The signal detection results show the multiple regression (\log_2 reporting odds ratio, x-axis) and significance ($-\log_{10}$ adjusted p-value, y-axis) for differentially expressed proteins. Red dots denote repressed proteins, green dots indicate activated proteins, and grey dots represent proteins that were not differentially expressed. The dotted lines indicate that the adjusted P-value=0.05. (C) Core differentially expressed protein interaction network diagram (a, BRD2; b, Hist1h4b; c, H3F3B; d, Gly). TMT, tandem mass tag; H4, hypoxia for 4 h; H/R2, H/R4 and H/R8, hypoxia/reoxygenation for 2, 4 or 8 h.

vesicle transport and exosome technology, such as Junb, Fstl1, Kert1, H3f3b, Ptgs2, Hif1 α , Hist1h4b, BRD2, Serpine1, and Glyr. The GO functional analysis revealed that these proteins can regulate vascular development, metal ion transport, and LDL lipid metabolism, suggesting their potential targets for reducing myocardial I/R injury (23-25). These results were consistent with the previous research. For instance, it has been demonstrated that Fstl1 is a cardioprotective factor regulated by Akt to reduce the myocardial I/R injury (26). In addition, inhibition of Fstl1 expression can aggravate cardiomyocyte apoptosis in myocardial H/R injury (27).

The BRD family serves as epigenetic ‘readers’ by recognizing acetylated histones and recruiting transcriptional regulator complexes to chromatin in order to regulate gene transcription (28). Due to the extensive role of the BRD family

in cardiovascular diseases, the present study explored the role of BRD2 in H/R4. When the BRD2 protein inhibitor dBET1 was performed in the H/R4 group, a decrease was found in cell viability and an increased level in LDH release and apoptosis in cardiomyocytes, indicating BRD2 may play a protective role in myocardial I/R injury. A previous study also found that the absence of BRD2 can decrease heart function and increase the mortality of conditional knock-out mice after pressure overload (29). In addition, the expression of BRD2 is lower in the adult heart than in the fetal heart, indicating its role in heart development and heart disease (7).

In addition, the present study discovered that proteins in the Nrf2/HO-1 signaling pathway reduced following the addition of dBET1, indicating that BRD2 plays a significant protective function through this pathway. Numerous studies

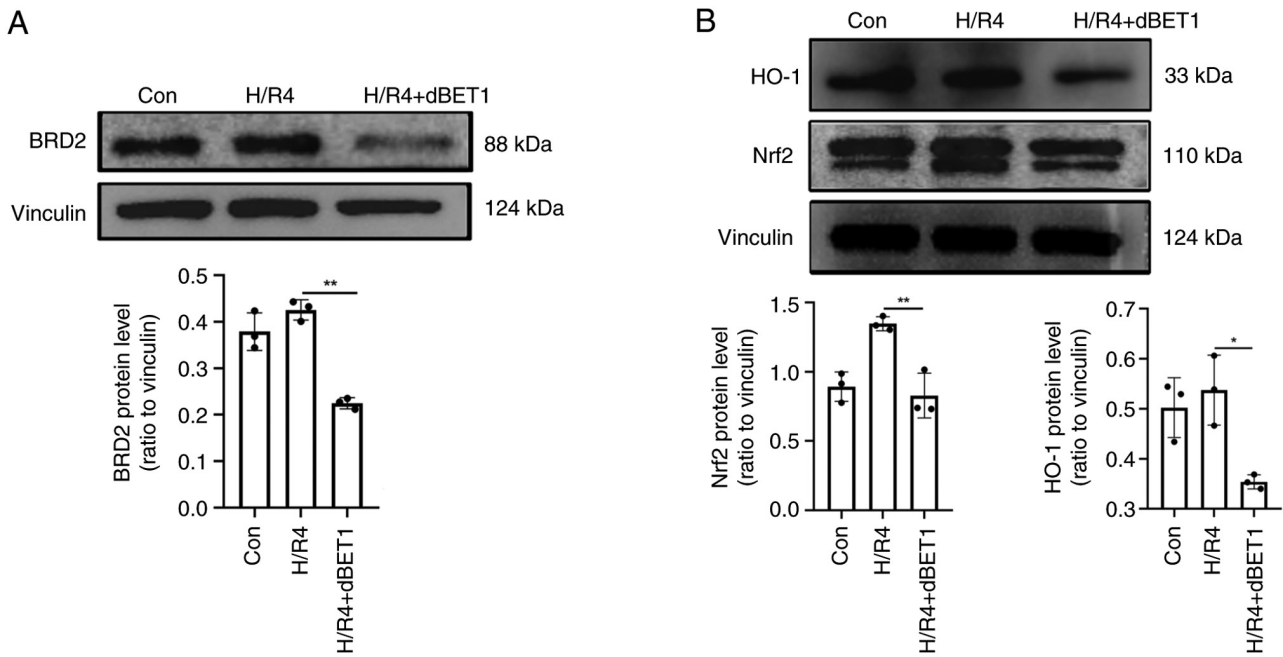


Figure 5. Effect of BRD2 downregulation on Nrf2/HO-1 signaling pathway in hypoxia-reoxygenation model. (A) Western blot analysis of the changes in the expression of Nrf2 and HO-1 by the downregulation of BRD2. Additional analysis indicated a trend for increased expression of BRD2 in hypoxia-reoxygenation for 4 h, while it significantly decreased after adding the inhibitor dBET1 ($P<0.01$). (B) There was a notable trend in the decrease in the Nrf2/HO-1 signaling pathway after adding the inhibitor dBET1 ($P<0.01$). For comparisons of these proteins' expression, one-way repeated-measures ANOVA followed by Tukey's post hoc test was used. $^*P<0.05$; $^{**}P<0.01$. Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, haem oxygenase-1; BRD2, bromodomain-containing protein 2; Con, control; H4, hypoxia for 4 h.

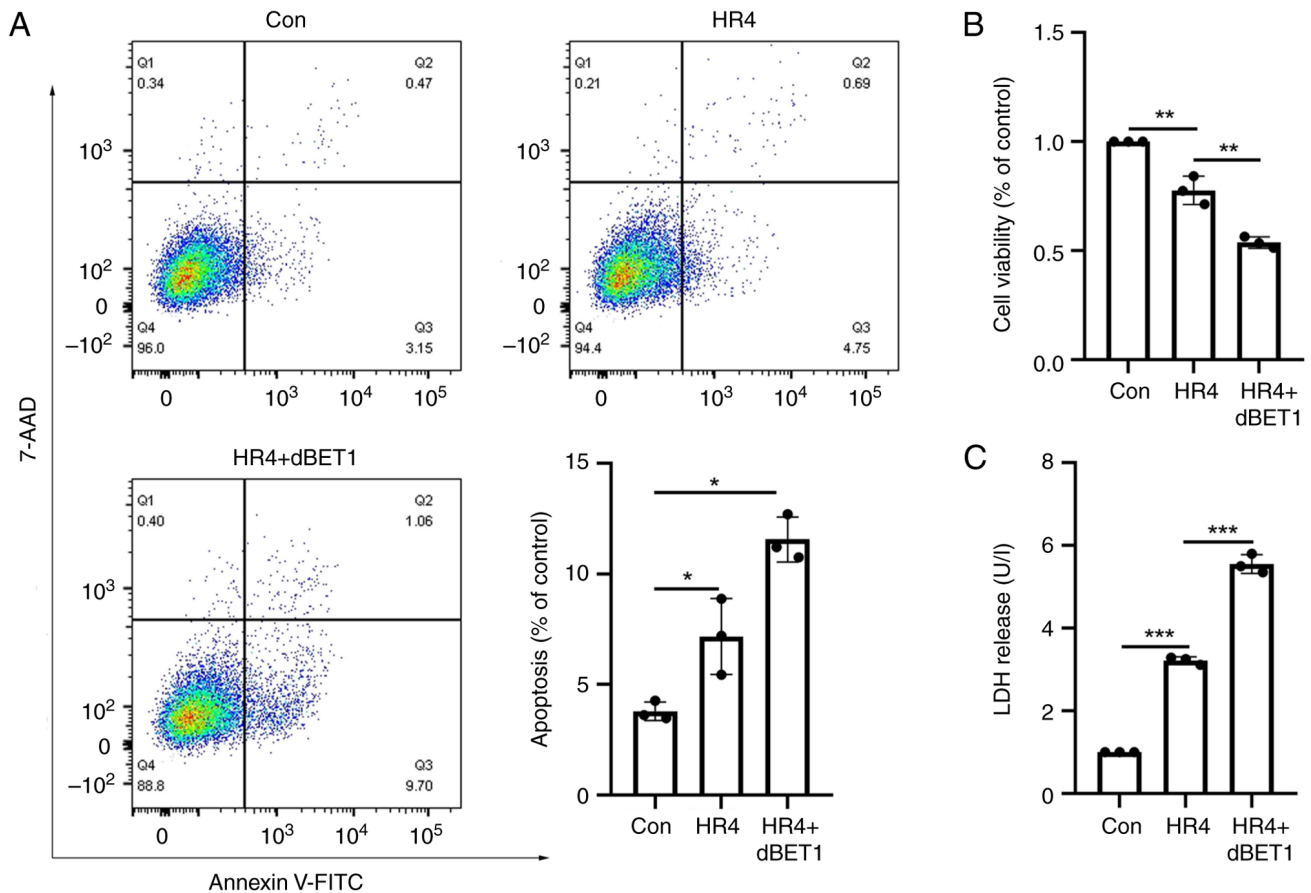


Figure 6. Effects of BRD2 inhibition on apoptosis, cell viability, and LDH release in hypoxia-reoxygenation model. (A) Flow cytometry was used to detect the changes in apoptosis after inhibiting BRD2. (B) After the dBET1 was added, the cell viability was decreased ($P<0.01$), but the ratio of apoptotic cells and (C) release of LDH was increased ($P<0.001$). The comparisons of apoptosis, cell viability, and LDH release was used one-way repeated-measures ANOVA followed by Tukey's post hoc test. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. LDH, lactose dehydrogenase; Con, control; H/R4, hypoxia/reoxygenation for 4 h.

have also established that this pathway plays a crucial role in the control of homeostasis by modifying calcium levels to inhibit autophagy, ferroptosis and clockophagy (30-32). Regarding cardiovascular illnesses, activation of the Nrf2/HO-1 pathway might enhance the prognosis following a myocardial infarction damage through nuclear translocation and overexpression of antioxidative genes (33). In addition, arrhythmias may be alleviated by activating the NO route through the Nrf2/HO-1 signaling axis (34). The activation of this pathway plays a crucial role in myocardial remodeling (35). The method by which BRD2 contributes to cardiac H/R damage through stimulation of the Nrf2/HO-1 signaling pathway may thus present a unique therapeutic target for the treatment of I/R injury in cardiomyocytes, as shown by the present findings.

Nevertheless, there were a few shortcomings associated with the present study. First, it was conducted in isolated cell experiments and therefore limits interpretations of the results. Second, BRD2-related agonists have not been found so far, so the role of the overexpression of BRD2 in I/R injury of cardiomyocytes has remained to be fully elucidated. Further pharmacological studies and animal studies are still warranted to validate the conclusions of the present study.

The present study was the first to show, to the best of the authors' knowledge, the sequential proteome alterations that occur in cardiomyocytes throughout the H/R process. It discovered a critical protective function for BRD2 in I/R damage to cardiomyocytes and demonstrated the connection to the Nrf2/HO-1 signaling pathway. It represented a new target for the treatment of I/R injuries of cardiomyocytes and provides an opportunity to initiate timely interventions following PCI procedures.

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Availability of data and materials

All datasets generated or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YL and LS conceived the present study. YL performed data curation. Conception and design was the responsibility of YL, YF and XX. YF and XX were responsible for the analysis and interpretation of data. LS and GT were responsible for supervision and project administration, and writing, reviewing and editing the manuscript. Visualization was the responsibility of XX. YL wrote the original draft of the manuscript. LS was

responsible for funding acquisition. YL, GT and LS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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