GSDMD-Mediated Cardiomyocyte Pyroptosis Promotes Myocardial I/R Injury

Huairui Shi,* Yang Gao,* Zhen Dong,* Ji'e Yang, Rifeng Gao, Xiao Li, Shuqi Zhang, Leilei Ma, Xiaolei Sun, Zeng Wang, Feng Zhang, Kai Hu, Aijun Sun, Junbo Ge

RATIONALE: Pyroptosis is a morphologically and mechanistically distinct form of cell death and is characterized by GSDMD (gasdermin D) or GSDME (gasdermin E)-mediated necrosis with excessive inflammatory factor release. Cardiomyocyte necrosis and inflammation play key roles in the pathophysiology of myocardial ischemia/reperfusion (I/R) injury. However, whether cardiomyocytes undergo pyroptosis and the underlying mechanism in myocardial I/R injury remain unclear.

OBJECTIVE: We aimed to investigate the role of pyroptosis in myocardial I/R injury.

METHODS AND RESULTS: In vivo and in vitro experiments were used to investigate pyroptosis of cardiomyocyte and the associated mechanisms during I/R injury. Wild-type, Myh6-Cre, and cardiomyocyte-specific GSDMD-deficient male mice were subjected to I/R. Human peripheral blood samples were collected from patients with acute ST-segment–elevation myocardial infarction or control patients at 0, 1, and 24 hours after percutaneous coronary intervention in our department. The serum levels of GSDMD were measured by ELISA. Hypoxia/reoxygenation induced cardiomyocyte pyroptosis and the release of mature IL (interleukin)-1β but not IL-1β, which mechanistically resulted from GSDMD cleavage by caspase-11 in cardiomyocytes. Furthermore, GSDMD gene deletion blocked hypoxia/reoxygenation-induced cardiomyocyte pyroptosis and IL-1β release. GSDMD and its pyroptosis-inducing N-terminal fragment were upregulated in myocardial tissues after I/R injury. Immunofluorescence analysis showed that GSDMD was mainly localized in cardiomyocytes. GSDMD deficiency in cardiomyocytes significantly reduced the I/R-induced myocardial infarct size. Moreover, increased GSDMD serum levels were detected in patients exhibiting I/R injury 1 hour after percutaneous coronary intervention for ST-segment–elevation myocardial infarction.

CONCLUSIONS: Our results show that GSDMD-mediated cardiomyocyte pyroptosis is a key event during myocardial I/R injury and that the caspase-11/GSDMD pathway may be essential to this process. Additionally, GSDMD inhibition significantly reduces cardiomyocyte pyroptosis and I/R-induced myocardial injury.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: caspase ■ cell death ■ inflammation ■ necrosis ■ pyroptosis

Myocardial infarction is one of the leading causes of death worldwide.1,2 Timely restoration of blood flow (reperfusion) in ischemic myocardial tissue remains the standard treatment for patients with myocardial infarction.3 However, a large amount of clinical evidence and experiments have confirmed that the beneficial effects of
reperfusion therapy could be counteracted to some extent by myocardial ischemia reperfusion (I/R) injury.4–7 Cardiomyocyte necrosis and inflammation play key roles in the pathophysiology of myocardial I/R injury. Myocardial I/R injury is mainly related to the death of terminally differentiated cardiomyocytes.8 The forms of cell death include apoptosis and necrosis, which occur during I/R injury. Apoptosis is considered to be a strictly controlled and well-established regulatory process, whereas cell necrosis has long been viewed as an uncontrollable mode of cell death.9–11 However, recent studies indicate that some types of cell necrosis, including necroptosis, pyroptosis, ferroptosis and oncosis, can also be regulated.12,13 Pyroptosis has distinct morphological features and is characterized by multiple bubble-like protrusions formed by GSDMD (gasdermin D) or GSDME (gasdermin E), cell swelling, membrane rupture, and the release of inflammatory factors such as IL-1β and IL-18.14,15

GSDMD belongs to the gasdermin family and is highly conserved in mammals. Gasdermins are composed of ≈480 amino acids and can be cleaved into gasdermin-N and gasdermin-C domains;18 GSDMD-N oligomerizes and perforates the plasma membrane to trigger pyroptosis and promote the secretion of IL-1β and IL-18 in macrophages.19,20 Previous studies have demonstrated the crucial role of pyroptosis in many human diseases, and excessive pyroptosis may be detrimental to the host.21,22 The occurrence of cardiomyocyte pyroptosis and the related mechanisms in myocardial I/R injury are not fully understood.

In this study, we demonstrated pyroptosis of cardiomyocytes undergoing oxidative stress, which plays a pivotal role in the pathogenesis of myocardial I/R injury. Cardiomyocyte conditional GSDMD knockout mice had significantly reduced I/R-induced myocardial infarct size. Knockdown of GSDMD blocked oxidative stress-induced pyroptosis of cardiomyocytes and IL-18 release in vitro. The CASP11-GSDMD pathway acts as an essential signaling pathway involved in pyroptosis of cardiomyocytes. What is more, our study found that GSDMD could be released during pyroptosis of cardiomyocytes into the periphery, and GSDMD levels were also increased in the serum of patients after reperfusion by percutaneous coronary intervention.

GSDMD and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| GSDMD        | gasdermin D |
| GSDME        | gasdermin E |
| H/R          | hypoxia/reoxygenation |
| I/R          | ischemia reperfusion |
| IL           | interleukin |
| MLKL         | mixed lineage kinase domain-like |
| NAC          | N-acetyl-L-cysteine |
| NLRP3        | NLR family pyrin domain containing 3 |
| PI           | propidium iodide |
| RIPK         | receptor-interacting protein kinase |

In this study, we demonstrated pyroptosis of cardiomyocytes undergoing oxidative stress, which plays a pivotal role in the pathogenesis of myocardial I/R injury. Cardiomyocyte conditional GSDMD knockout mice had significantly reduced I/R-induced myocardial infarct size. Knockdown of GSDMD blocked oxidative stress-induced pyroptosis of cardiomyocytes and IL-18 release in vitro. The CASP11-GSDMD pathway acts as an essential signaling pathway involved in pyroptosis of cardiomyocytes. What is more, our study found that GSDMD could be released during pyroptosis of cardiomyocytes into the periphery, and GSDMD levels were also increased in the serum of patients after reperfusion by percutaneous coronary intervention.

GSDMD and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| GSDMD        | gasdermin D |
| GSDME        | gasdermin E |
| H/R          | hypoxia/reoxygenation |
| I/R          | ischemia reperfusion |
| IL           | interleukin |
| MLKL         | mixed lineage kinase domain-like |
| NAC          | N-acetyl-L-cysteine |
| NLRP3        | NLR family pyrin domain containing 3 |
| PI           | propidium iodide |
| RIPK         | receptor-interacting protein kinase |

In this study, we demonstrated pyroptosis of cardiomyocytes undergoing oxidative stress, which plays a pivotal role in the pathogenesis of myocardial I/R injury. Cardiomyocyte conditional GSDMD knockout mice had significantly reduced I/R-induced myocardial infarct size. Knockdown of GSDMD blocked oxidative stress-induced pyroptosis of cardiomyocytes and IL-18 release in vitro. The CASP11-GSDMD pathway acts as an essential signaling pathway involved in pyroptosis of cardiomyocytes. What is more, our study found that GSDMD could be released during pyroptosis of cardiomyocytes into the periphery, and GSDMD levels were also increased in the serum of patients after reperfusion by percutaneous coronary intervention.

GSDMD and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| GSDMD        | gasdermin D |
| GSDME        | gasdermin E |
| H/R          | hypoxia/reoxygenation |
| I/R          | ischemia reperfusion |
| IL           | interleukin |
| MLKL         | mixed lineage kinase domain-like |
| NAC          | N-acetyl-L-cysteine |
| NLRP3        | NLR family pyrin domain containing 3 |
| PI           | propidium iodide |
| RIPK         | receptor-interacting protein kinase |

In this study, we demonstrated pyroptosis of cardiomyocytes undergoing oxidative stress, which plays a pivotal role in the pathogenesis of myocardial I/R injury. Cardiomyocyte conditional GSDMD knockout mice had significantly reduced I/R-induced myocardial infarct size. Knockdown of GSDMD blocked oxidative stress-induced pyroptosis of cardiomyocytes and IL-18 release in vitro. The CASP11-GSDMD pathway acts as an essential signaling pathway involved in pyroptosis of cardiomyocytes. What is more, our study found that GSDMD could be released during pyroptosis of cardiomyocytes into the periphery, and GSDMD levels were also increased in the serum of patients after reperfusion by percutaneous coronary intervention.

GSDMD and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| GSDMD        | gasdermin D |
| GSDME        | gasdermin E |
| H/R          | hypoxia/reoxygenation |
| I/R          | ischemia reperfusion |
| IL           | interleukin |
| MLKL         | mixed lineage kinase domain-like |
| NAC          | N-acetyl-L-cysteine |
| NLRP3        | NLR family pyrin domain containing 3 |
| PI           | propidium iodide |
| RIPK         | receptor-interacting protein kinase |

In this study, we demonstrated pyroptosis of cardiomyocytes undergoing oxidative stress, which plays a pivotal role in the pathogenesis of myocardial I/R injury. Cardiomyocyte conditional GSDMD knockout mice had significantly reduced I/R-induced myocardial infarct size. Knockdown of GSDMD blocked oxidative stress-induced pyroptosis of cardiomyocytes and IL-18 release in vitro. The CASP11-GSDMD pathway acts as an essential signaling pathway involved in pyroptosis of cardiomyocytes. What is more, our study found that GSDMD could be released during pyroptosis of cardiomyocytes into the periphery, and GSDMD levels were also increased in the serum of patients after reperfusion by percutaneous coronary intervention.

GSDMD and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| GSDMD        | gasdermin D |
| GSDME        | gasdermin E |
| H/R          | hypoxia/reoxygenation |
| I/R          | ischemia reperfusion |
| IL           | interleukin |
| MLKL         | mixed lineage kinase domain-like |
| NAC          | N-acetyl-L-cysteine |
| NLRP3        | NLR family pyrin domain containing 3 |
| PI           | propidium iodide |
| RIPK         | receptor-interacting protein kinase |

In this study, we demonstrated pyroptosis of cardiomyocytes undergoing oxidative stress, which plays a pivotal role in the pathogenesis of myocardial I/R injury. Cardiomyocyte conditional GSDMD knockout mice had significantly reduced I/R-induced myocardial infarct size. Knockdown of GSDMD blocked oxidative stress-induced pyroptosis of cardiomyocytes and IL-18 release in vitro. The CASP11-GSDMD pathway acts as an essential signaling pathway involved in pyroptosis of cardiomyocytes. What is more, our study found that GSDMD could be released during pyroptosis of cardiomyocytes into the periphery, and GSDMD levels were also increased in the serum of patients after reperfusion by percutaneous coronary intervention.

GSDMD and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| GSDMD        | gasdermin D |
| GSDME        | gasdermin E |
| H/R          | hypoxia/reoxygenation |
| I/R          | ischemia reperfusion |
| IL           | interleukin |
| MLKL         | mixed lineage kinase domain-like |
| NAC          | N-acetyl-L-cysteine |
| NLRP3        | NLR family pyrin domain containing 3 |
| PI           | propidium iodide |
| RIPK         | receptor-interacting protein kinase |

In this study, we demonstrated pyroptosis of cardiomyocytes undergoing oxidative stress, which plays a pivotal role in the pathogenesis of myocardial I/R injury. Cardiomyocyte conditional GSDMD knockout mice had significantly reduced I/R-induced myocardial infarct size. Knockdown of GSDMD blocked oxidative stress-induced pyroptosis of cardiomyocytes and IL-18 release in vitro. The CASP11-GSDMD pathway acts as an essential signaling pathway involved in pyroptosis of cardiomyocytes. What is more, our study found that GSDMD could be released during pyroptosis of cardiomyocytes into the periphery, and GSDMD levels were also increased in the serum of patients after reperfusion by percutaneous coronary intervention.
by GSDMD- or GSDME-induced plasma membrane rupture.\textsuperscript{13,14} To determine whether hypoxia/reoxygenation (H/R) induced cardiomyocyte pyroptosis, we performed continuous live cell imaging experiments to document the morphological changes in adult cardiomyocytes. As shown in Figure 1A and Movie I in the Data Supplement, cardiomyocytes produced large numbers of balloon-shaped vesicles and took up propidium iodide (PI), which are typical characteristics of pyroptosis,\textsuperscript{13,14} as well as exhibited decreased ATP levels and the loss of cell membrane integrity (as assessed by LDH [lactate dehydrogenase] release) compared with those of the nontreatment group Figure 1F through 1I. Notably, cardiomyocyte H/R induced the release of the inflammatory factor IL-1β, which is associated with pyroptosis, in the cell culture supernatant, but there was no IL-1β release (not detected in the cell culture supernatant). To further confirm cardiomyocyte pyroptosis, we examined the key pyroptotic proteins GSDMD and GSDME. Immunoblot analysis revealed significantly increased levels of GSDMD and GSDMD-N after H/R in adult cardiomyocytes in a time-dependent manner, but GSDME showed no statistical difference (Figure 1B and 1C). Consistent with these observations, immunofluorescence revealed that GSDMD was also expressed in adult cardiomyocytes (Figure 1D).

To fully verify the key role of GSDMD in cardiomyocyte pyroptosis, we generated GSDMD-CKO mice and isolated adult and neonatal cardiomyocytes from the heart. The depletion of GSDMD protein in cardiomyocytes was verified by Western blotting (Figure I in the Data Supplement). In adult cardiomyocytes, the absence of GSDMD markedly blocked H/R-induced cardiomyocyte pyroptosis (Figure 1E, Movie II in the Data Supplement). Consistent with these observations, deletion of the GSDMD gene abolished cardiomyocyte pyroptosis, as evidenced by the loss of cell membrane integrity (as assessed by PI-positive cardiomyocytes and LDH release in Figure 1F and 1G), decreased ATP levels (Figure 1H) and IL-18 release (Figure 1I). Moreover, H/R-induced neonatal mouse ventricular cardiomyocytes (Figure IIA and Movie III in the Data Supplement) and AC16 cells pyroptosis (Movies IV and V in the Data Supplement) that were subjected to hypoxia followed by reoxygenation. Immunofluorescence results showed that GSDMD was also expressed in neonatal mouse ventricular cardiomyocytes (Figure IIB in the Data Supplement). Deletion of the GSDMD gene abolished pyroptosis in neonatal mouse ventricular cardiomyocytes (Figure IIC and Movie VI in the Data Supplement), as evidenced by PI-positive cardiomyocytes and LDH release (Figure IID and IIE in the Data Supplement), ATP levels (Figure IIF in the Data Supplement) and IL-18 release (Figure II G in the Data Supplement) in neonatal mouse ventricular cardiomyocytes. These data showed that H/R could induce cardiomyocyte pyroptosis, and GSDMD played a key role in this process.

The N-Terminal Fragment of GSDMD Is Required for Cardiomyocyte Pyroptosis

GSDMD-N oligomerizes and perforates the plasma membrane to trigger pyroptosis.\textsuperscript{16,17} To further confirm the effect of GSDMD on cardiomyocyte pyroptosis, we overexpressed full-length GSDMD, GSDMD-N, or GSDMD-C in adult cardiomyocytes (Figure 2A through 2C). Overexpression of GSDMD-N in cultured cardiomyocytes led to robust pyroptosis, while similar effects were not observed after the overexpression of full-length GSDMD or GSDMD-C in adult cardiomyocytes (Figure 2D through 2G). These findings showed that the N-terminal fragment was the direct effector of GSDMD and confirmed the key role of GSDMD in cardiomyocyte pyroptosis.

The CASP11-GSDMD-N Pathway Mediates Pyroptosis in H/R-Stimulated Cardiomyocytes

Recent studies have shown that GSDMD cleavage is mediated by caspase-1/11, which can specifically cleave the linker between the GSDMD-N and GSDMD-C domains in macrophages.\textsuperscript{12} To further define how GSDMD was cleaved to generate GSDMD-N and execute pyroptosis in cardiomyocytes during H/R, the expression of caspase-1 and caspase-11 was measured by immunoblotting. Our results showed that H/R (0.5/24 hours) selectively activated caspase-11 (Figure 3A) but not caspase-1 or the canonical inflammasome (Figure 3B). Consistent with the immunoblot analysis, the knockdown of caspase-11 by adenoviral transfection markedly reduced the generation of GSDMD-N, while caspase-1 knockdown had no significant effect on GSDMD-N (Figure 3B). Consistent with the immunoblot analysis, the knockdown of caspase-11 also abrogated pyroptosis in cardiomyocytes, as indicated by PI uptake (Figure 3C and 3D), LDH release (Figure 3E), cellular ATP levels (Figure 3F), and IL-18 secretion (Figure 3G). Moreover, to confirm the interaction between GSDMD and caspase-11, flag-tagged GSDMD was expressed in adult mouse cardiomyocytes. Coimmunoprecipitation revealed interactions between caspase-11 and GSDMD in adult cardiomyocytes subjected to H/R (0.5/24 hours; Figure VIA in the Data Supplement). In addition, immunofluorescence costaining of GSDMD and caspase-11 in adult cardiomyocytes after H/R (0.5/24 hours) confirmed the colocalization of GSDMD and caspase-11 in cardiomyocytes (Figure VIB in the Data Supplement), while GSDME was no colocalized with caspase-11 (Figure VII in the Data Supplement). Taken together, these results suggested that the caspase-11-GSDMD pathway mediated H/R-induced cardiomyocyte pyroptosis.
Figure 1. Hypoxia/reoxygenation (H/R) induces cardiomyocyte pyroptosis through GSDMD (gasdermin D) activation.

Adult cardiomyocytes from the ventricles of Myh6-Cre GSDMD +/+ mice (controls) or GSDMD-CKO (cardiomyocyte-specific GSDMD-deficient) male mice were isolated, and (A) H/R for 0.5/24 h induced the pyroptotic morphological manifestations of prominent bubble-like formations, as well as propidium iodide (PI)-positive staining of adult cardiomyocytes (black indicates necrotic cardiomyocytes and prominent bubble-like formations). B and C, GSDMD (n=5) and GSDME (gasdermin E) protein levels (n=5) and cleavage were (Continued)
Pyroptosis of Cardiomyocytes Stimulated by I/R Injury In Vivo

To investigate the involvement of myocardial pyroptosis in I/R injury in vivo, we generated a mouse myocardial I/R injury model (30 minutes of ischemia followed by reperfusion for 3, 12, or 24 hours). The expression of related proteins was determined by Western blot analysis of protein samples extracted from the left ventricular myocardium. The results showed that GSDMD and GSDMD-N levels were significantly upregulated in the myocardium after 3 hours of reperfusion and further increased after 24 hours of reperfusion (Figure 4A), while GSDME was no statistical difference (Figure 4B). Moreover, the levels of caspase-11 were markedly upregulated after 24 hours of reperfusion (Figure 4C). In addition, immunofluorescence analysis revealed that GSDMD and caspase-11 accumulation also mainly occurred in cardiomyocytes (Figure 4D). GSDME rarely expressed during this process (Figure VIII in the Data Supplement). Taken together, these results suggest that caspase-11- and GSDMD-mediated cardiomyocyte pyroptosis is involved in myocardial I/R injury.

GSDMD Deficiency Reduced I/R-Induced Myocardial Injury In Vivo

To further confirm the involvement of GSDMD-dependent myocardial pyroptosis in vivo, we generated cardiac-specific knockout (GSDMDfl/fl; Creα-MHC [GSDMD-CKO]) male mice, and myocardial I/R injury was induced as described above. These mice had depleted levels of GSDMD protein in the myocardium but not in other tissues tested (Figure IX in the Data Supplement). The infarct size/area at risk ratio of GSDMD-CKO mice (19.8%) was significantly decreased following 30 minutes of ischemia and 24 hours of reperfusion compared with that of Myh6-Cre mice (36.5%), (P<0.05; Figure 5A through 5C). Consistent with the triphenyl tetrazolium chloride/Evans blue staining results, I/R-induced LDH (Figure 5D) release and myocardial necrosis as shown by Evans blue dye-positive staining (Evans blue dye uptake) after I/R were also reduced in the GSDMD-CKO mice (Figure 5E and 5F). However, there were no statistical difference in apoptotic cardiomyocytes, as shown by TUNEL staining (Figure X in the Data Supplement). Collectively, our results clearly demonstrated the role of GSDMD-mediated pyroptosis in myocardial I/R injury.

Oxidative Stress Is a Key Factor in the Induction of Cardiomyocyte Pyroptosis During I/R Injury

Oxidative stress is thought to be one of the main pathogenic mediators of myocardial I/R injury. \( \text{H}_2\text{O}_2 \) is widely used to mimic the pathophysiological effects of oxidative stress during reperfusion. In our study, similar to H/R, \( \text{H}_2\text{O}_2 \) treatment induced cardiomyocyte pyroptosis (Figure 6A), as indicated by increased numbers of PI-positive cardiomyocytes and decreased cellular ATP levels and LDH release (Figure 6B through 6D). These changes were attenuated by knockout of GSDMD. Furthermore, GSDMD-N was activated in cardiomyocytes stimulated with \( \text{H}_2\text{O}_2 \) (Figure 6E).

N-acetyl-L-cysteine (NAC) is commonly used as an antioxidant to eliminate the effect of oxidative stress. Our results demonstrated that NAC decreased the H/R-induced upregulation of GSDMD and GSDMD-N protein levels in adult mouse cardiomyocytes (Figure XI in the Data Supplement). In addition, Myh6-Cre, GSDMD+/− and GSDMD-CKO mice treated with PBS or NAC (400 mg/kg) were subjected to myocardial I/R injury (30 minutes of ischemia followed by reperfusion for 24 hours). The results showed that the infarct size/area at risk ratios and serum LDH concentrations were significantly reduced in Myh6-Cre mice treated with NAC (Figure XII in the Data Supplement). Moreover, the protein levels of GSDMD and caspase-11 were markedly reduced after 24 hours of reperfusion in the hearts of mice treated with NAC (Figure XIII in the Data Supplement). Taken together, these results suggested that oxidative stress-induced cardiomyocyte pyroptosis via a GSDMD-dependent pathway and that the elimination of oxidative stress alleviated GSDMD-mediated pyroptosis induced by myocardial I/R injury.

GSDMD Can be Released From Pyroptotic Cardiomyocytes

Interestingly, GSDMD release in the culture supernatants of cardiomyocytes that underwent H/R was measured by Western blotting and ELISA (Figure 7A and 7B). GSDMD levels were measured in the serum of ST-segment–elevation myocardial infarction and stable coronary artery disease (CAD) patients at 0, 1, and 24 hours after percutaneous coronary intervention by ELISA. Serum GSDMD levels were also...
Figure 2. GSDMD (gasdermin D) triggers cardiomyocyte pyroptosis due to the intrinsic pore-forming activity of its N-terminal domain.

**A–C.** The full-length GSDMD (OV-GSDMD-FL, n=5), the GSDMD-N (OV-GSDMD-N, n=5) and GSDMD-C (OV-GSDMD-C, n=5) fragments, was transiently expressed in adult cardiomyocytes via plasmid transfection. The target band was first normalized with β-actin and then calculated as fold changes vs control groups. **D.** Hoechst 33342 (blue) and PI were added to detect the loss of plasma membrane integrity, and fluorescent images were obtained by confocal microscopy. Black arrows indicate pyroptosis in cardiomyocytes and prominent bubble-like formations. **E (n=5),** Propidium iodide (PI)-positive and total (Hoechst-positive) cells were counted in 5 randomly selected fluorescence microscopic visual fields. **F and G,** Cellular ATP levels (n=5) and LDH (lactate dehydrogenase) release (n=5) in the supernatants of adult mouse cardiac myocytes (subjected to hypoxia/reoxygenation [H/R] 0.5/24 h, LDH and ATP levels) were calculated as fold changes vs control group. Vector group (control plasmid), OV-GSDMD-FL (overexpressing plasmid pcDNA3.1-GSDMD), OV-GSDMD-N (overexpressing plasmid pcDNA3.1-GSDMD-N), OV-GSDMD-C (overexpressing plasmid pcDNA3.1-GSDMD-C). The data are expressed as the means±SEMs. P values were determined by using 1-way ANOVA with Bonferroni multiple comparisons test (**A, B, and C,** P values adjusted for 5 comparisons; **E, F, and G,** P values adjusted for 6 comparisons). NS indicates not significant.
Figure 3. CASP11 (caspase-11) cleaves GSDMD (gaspermin D) in cardiomyocytes undergoing hypoxia/reoxygenation (H/R)-induced pyroptosis.

Cardiomyocytes were stimulated with H/R for the indicated times. A (n=5), Western blot (WB) analysis was performed to measure caspase-11. Representative WB analysis of GSDMD and GSDMD-N in cardiomyocytes infected with Ad-β-gal (used as the control, n=5), Ad-CASP11 or Ad-CASP1 for 48 h and then treated with H/R for 0.5/24 h. C, Propidium iodide (PI) was added to detect the loss of plasma membrane integrity, and fluorescent images were obtained by confocal microscopy. D–G (n=5), LDH (lactate dehydrogenase) release, cellular ATP levels, and IL-18 levels in supernatants as measured by ELISA (LDH and ATP levels were calculated as fold changes vs control group). The data are expressed as the means±SEMs. Fold changes in protein levels in the treatment groups compared with the control groups were determined (the target band was first normalized with β-actin and then calculated as fold changes vs control groups). P values were determined by using 1-way ANOVA with Bonferroni multiple comparisons test (A and B, P values adjusted for 4 comparisons), 2-way ANOVA with Bonferroni multiple comparisons test (D–G, P values adjusted for 4 comparisons). NS indicates not significant; and WT, wild-type.

significantly higher after percutaneous coronary intervention in patients with ST-segment–elevation myocardial infarction than in age-matched stable CAD patients (Figure 7C). Collectively, these findings suggested that GSDMD could be released from pyroptotic cardiomyocytes in a time-dependent manner.

DISCUSSION

To the best of our knowledge, this is the first report to describe cardiomyocyte pyroptosis and the associated signaling pathway in myocardial I/R injury. The present study demonstrated that H/R could induce cardiomyocyte...
pyroptosis. Moreover, GSDMD knockdown significantly reduced cardiomyocyte pyroptosis, which is a driving force in myocardial I/R injury and alleviated I/R-induced myocardial injury in mice. Pyroptosis has been previously considered to be a mode of caspase-1-mediated death in monocytes; however, we found that cardiomyocyte pyroptosis was mainly regulated via the caspase-11/GSDMD signaling pathway under H/R conditions, and this process was accompanied by the release of mature IL-18 but not IL-1β.

Apoptosis and necrosis are forms of cell death that are involved in the process of myocardial I/R injury. 

Apoptosis is characterized by cell shrinkage, membrane blebbing, and the formation of apoptotic bodies. Apoptosis is usually noninflammatory in nature, is initiated by caspases -2, -8, and -9 and requires effector caspases -3, -6, and -7. Necroptosis is a form of necrosis that is similar to cellular eruption and involves the activation of the RIPK (receptor-interacting protein kinase) 1/RIPK3/MLKL (mixed lineage domain-like) pathway. Pyroptosis is a form of necrosis that is highly inflammatory and characterized by various morphological and mechanistic distinctions. The morphological features of pyroptosis include the formation of bubble-like protrusions, the formation of pores in the plasma membrane, cell swelling, membrane rupture, and the release of inflammatory factors, such as IL-1β and IL-18. Our results confirm that cardiomyocytes undergo pyroptosis in response to myocardial I/R injury. Genetic screening studies have identified GSDMD as a
Shi et al Cardiomyocyte Pyroptosis Aggravates MI/R Injury

**Key mediator of pore formation in macrophages during pyroptosis.** Our in vitro experiments revealed that H/R or oxidative stress caused cardiomyocyte pyroptosis, resulting in the production of large numbers of balloon-shaped vesicles, which are characteristic of pyroptosis, and increased cardiomyocyte necrosis, as well as the release of LDH and IL-18. GSDMD deficiency reversed these pyroptotic effects in cardiomyocytes in response to H/R or oxidative stress. Our in vivo experiments showed that the levels of pyroptosis-associated proteins, including GSDMD, GSDMD-N and caspase-11, were markedly increased after myocardial I/R injury. Furthermore, GSDMD deficiency significantly reduced I/R-induced myocardial infarct size. Our findings suggest that cardiomyocyte pyroptosis is a major determinant of the extent of myocardial I/R injury and that GSDMD may be a potential therapeutic target to alleviate myocardial I/R injury. Sex might affect I/R-induced myocardial injury in vivo, as female mice, rats, or rabbits showed reduced I/R injury compared with males in several experiments.34,35 The difference can be partly explained by the impact of estrogen and estrogen receptors.35,36 Therefore, to avoid the impact of female sex on the experimental results, only male mice were used in animal experiment. Future studies are warranted to observe if our finding in male mice could be validated in female mice or not.

**GSDMD plays an effector role in pyroptosis.** Our results indicate that GSDMD gene deletion significantly suppresses H/R-induced cardiomyocyte pyroptosis. Specifically, GSDMD-N is sufficient to drive pyroptosis, regardless of the cellular system. Furthermore, overexpression of GSDMD-N but not full-length GSDMD causes pyroptosis in cardiomyocytes. Our findings are consistent with those of a previous study, in

---

**Figure 5. GSDMD (gasdermin D) deficiency in cardiomyocytes blocks myocardial ischemia/reperfusion (I/R) injury.**

Wild-type (WT; n=10 per group), Myh6-Cre GSDMD +/+ (n=10 per group), GSDMDF/F (n=10 per group), and GSDMD-CKO (cardiomyocyte-specific GSDMD-deficient; n=10 mice per group) mice were subjected to I/R injury (30 min of ischemia/24 h of reperfusion). A, Representative photographs of cross-sections stained with triphenyl tetrazolium chloride (TTC) and Evans blue (EB) to determine the extent of I/R injury. B and C, The ratios (right) of infarct size (IF, n=10) to area at risk (AAR, n=10) in the hearts. D, Serum LDH (lactate dehydrogenase) levels (n=10) of mice with I/R injury (30 min of ischemia/24 h of reperfusion). E, Representative photographs and quantitative analysis of myocardial Evans blue dye uptake, which shows infarcted cardiomyocytes; viable cardiomyocytes were labeled by cTnT antibodies in WT, Myh6-Cre, GSDMDF/F, and GSDMD-CKO (cardiomyocyte-specific GSDMD-deficient) mice (n=10 mice per group) subjected to I/R injury. The data are expressed as the means±SEM, the normal distribution was checked by the Shapiro-Wilk test, all P values were >0.05 indicated that the data were approximately normally distributed for each group. P values were determined by using 1-way ANOVA with Bonferroni multiple comparisons test (B, C, and F, P values adjusted for 3 comparisons), 2-way ANOVA with Bonferroni multiple comparisons test (D). cTnT indicates cardiac troponin T; and NS, not significant.
which the N-terminal domain of GSDMD was demonstrated to be essential for pyroptosis. GSDMD is a substrate of inflammatory caspases; however, the cleavage mechanism of GSDMD activation is not well defined in cardiomyocytes. Therefore, we wanted to determine whether GSDMD was directly cleaved by inflammatory caspases. Our study revealed that caspase-11 expression was strongly induced in cardiomyocyte pyroptosis under H/R conditions. The inflammasome-related proteins NLRP3 (NLR family pyrin domain containing 3) and apoptosis-associated speck-like protein containing are both required for caspase-1 activation; however, we found that these canonical inflammasome components were hardly expressed in cardiomyocytes, which was consistent with previous findings. Thus, our results suggest that caspase-11 may be the only pathway available in cardiomyocytes to trigger pyroptosis. Our data show that caspase-11 knockout prevents the formation of GSDMD-N and subsequent pyroptosis in cardiomyocytes, whereas caspase-1 knockout does not affect GSDMD-N availability. Thus, caspase-11/GSDMD-dependent pyroptosis in cardiomyocytes in response to H/R may be the main mechanism triggered by the noncanonical inflammasome in the context of myocardial I/R injury. In addition, oxidative...
stress is a major apoptotic and necrotic stimulus in the progression of I/R injury. Similar to H/R, H2O2 treatment (mimicking the pathophysiological effects of oxidative stress) also induced cardiomyocyte pyroptosis. NAC (an antioxidant that eliminates the effects of oxidative stress) decreased H/R-induced cardiomyocyte pyroptosis and upregulation of GSDMD-N and caspase11 protein levels in adult mouse cardiomyocytes. Taken together, these results suggested that oxidative stress may induce the interaction between C11 and GSDMD during pyroptosis in H/R-induced cardiomyocytes.

A large amount of evidence indicates the importance of inflammation in the pathophysiology of myocardial I/R injury. IL-18 is a pleiotropic cytokine that belongs to the IL-1 family, and its expression is upregulated in various immune and infectious diseases, as well as in response to myocardial I/R. IL-18 further amplifies the inflammatory cascade by inducing additional cytokines, adhesion molecules, and chemokines. Neutralization of IL-18 significantly attenuates I/R-induced tissue damage in vivo. Our findings also showed that H/R markedly increased the expression of the proinflammatory cytokine IL-18 and its release into the cell culture supernatant, but these effects were not observed for IL-1β. Additionally, GSDMD deletion blocked I/R-induced IL-18 release from cardiomyocytes. Our findings are consistent with those of Chandrasekar et al., who showed that IL-18 mRNA and protein expression levels were upregulated and that the release of IL-18 into the cell culture supernatant was increased by oxidative stress in cardiomyocytes. IL-1β is the master switch in the inflammatory response and plays an important role in inflammatory cardiovascular diseases, however, IL-1β expression is low in cardiomyocytes under oxidative stress. Our findings are consistent with the results of Kawaguchi et al., who showed that the expression of IL-1β was mainly derived from fibroblasts and that IL-1β was not significantly altered in cardiomyocytes under oxidative stress conditions. Therefore, cardiomyocyte pyroptosis and release of the proinflammatory cytokine IL-18 may activate cardiac fibroblasts to induce the secondary production of cytokines.

The most important result of our study is that GSDMD is released into the peripheral culture media after cardiomyocyte pyroptosis in response to H/R. Additionally, GSDMD levels are detectable after percutaneous coronary intervention and I/R injury in patient sera. These
levels are significantly increased 1 hour after percutaneous coronary intervention compared with the preoperative levels and those of control patients without coronary artery disease, as determined by coronary angiography. These findings suggest that serum GSDMD levels may also be used for the diagnosis of myocardial I/R injury. However, further studies are warranted to investigate the details and elucidate the underlying associated mechanisms.

In conclusion, our data demonstrate that GSDMD-induced pyroptosis plays a pivotal role in the pathogenesis of myocardial I/R injury and that cardiomyocyte pyroptosis is mainly regulated via the caspase-11/GSDMD signaling pathway under oxidative stress conditions (Figure 8). These findings provide new insights into the treatment of myocardial I/R injury. Furthermore, GSDMD may be a potential biomarker and therapeutic target for the identification and treatment of cardiomyocyte pyroptosis and subsequent myocardial I/R injury.
Shi et al Cardiomyocyte Pyroptosis Aggravates MI/R Injury

Cardiomyocyte Pyroptosis Aggravates MI/R Injury

Shi et al. Cardiomyocyte Pyroptosis Aggravates MI/R Injury

24. Shiokawa D, Maruta H, Tanuma S. Inhibitors of poly(ADP-ribose) polymerase suppress nuclear fragmentation and apoptotic-body formation during apoptosis in HL-60 cells. FEMS Lett. 1997;413:99–103. doi: 10.1016/S0168-6444(97)00087-9

25. Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun. 2005;73:1907–1916. doi: 10.1128/IAI.73.1907-1916.2005

26. He S, Wang L, Mao L, Wang T, Du F, Zhao L, Wang X. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell. 2009;137:1109–1111. doi: 10.1016/j.cell.2009.05.021

27. Sendler M, Mayerle J, Lerch MM. Necrosis, apoptosis, pyroptosis—It matters how acinar cells die during pancreatitis. Cell Mol Gastroenterol Hepatol. 2016;2:407–408. doi: 10.1016/j.jcmgh.2016.05.007

28. Shi J, Gao W, Shao F. Pyroptosis: gasdermin-mediated programmed necrotic cell death. Trends Biochem Sci. 2017;42:245–254. doi: 10.1016/j.tibs.2016.10.004

29. Bergsaker H, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. Nat Rev Microbiol. 2007;9:99–109. doi: 10.1038/nrmicro2070

30. Ding J, Wang L, Xie Y, Liu W, Wang Z, Shi J, Sun H, Wang DC, Shao F. Pore-forming activity and structural autodigestion of the gasdermin family. Nature. 2016;535:111–116. doi: 10.1038/nature18590

31. Storgi L, Rühli S, Muñihili P, Eiterevic J, Heiligen H, Stahlberg H, Farady CJ, Müller DJ, Braz P. Filler S. GSDMD membrane pore formation constitutes the mechanistic pyroptotic cell death. EMBO J. 2016;35:1765–1778. doi: 10.15252/embj.201694696

32. Liu X, Zhang Z, Ruan J, Pan Y, Magupalli VG, Wu H, Lieberman J. Inflammation-activated gasdermin D causes pyroptosis by forming membrane pores. Nature. 2016;535:153–158. doi: 10.1038/nature18629

33. Aglietti RA, Estevez A, Gupta A, Ramirez MG, Liu PS, Kayagaki N, Ciferri C, Dixit VM, Duerer EC. GSDMD p30 cleaved by caspase-11 during pyroptosis forms pores in membranes. Proc Natl Acad Sci U S A. 2016;113:7858–7863. doi: 10.1073/pnas.1601661113

34. Buoma W, Noma M, Kanemoto S, Matsubara M, Leshower BG, Himron R, Gorman JH III, Gorman RC. Sex-related resistance to myocardial ischaemia-reperfusion injury is associated with high constitutive ARC expression. Am J Physiol Heart Circ Physiol. 2010;298:H1510–H1517. doi: 10.1152/ajpheart.01209.2009

35. Murphy E, Steenbergen C. Gender-based differences in mechanisms of protection in myocardial ischaemia-reperfusion injury. Cardiovasc Res. 2007;75:478–486. doi: 10.1016/j.cardiores.2007.07.003

36. Ostadal B, Ostadal P. Sex-based differences in cardiac ischaemic injury and protection: therapeutic implications. Br J Pharmacol. 2014;171:541–554. doi: 10.1111/bjp.12270

37. He WT, Wan H, Hu L, Chen P, Wang X, Huang Z, Yang ZH, Zhong CQ, Han J. Gasdermin D is an executor of pyroptosis and required for myocardial protection. Circ Res. 2015;21:1285–1298. doi: 10.1161/CIRCRESAHA.115.004107

38. Gaidt MM, Hornung V. Pore formation by GSDMD is the effector mechanism of pyroptosis. EMBO J. 2016;35:2167–2169. doi: 10.15252/embj.201695415

39. Kawaguchi M, Takahashi M, Hata T, Kashima Y, Usui F, Morimoto H, Izawa T, Nakazawa R, Frangogiannis NG. The role of IL-1 in the pathogenesis of heart disease: mechanisms and significance. Annu Rev Physiol. 2010;72:19–44. doi: 10.1146/annurev.physiol.010908.163111

40. Whelan RS, Kaplaniski V, Kitis RN. Cell death in the pathogenesis of heart disease: mechanisms and significance. Annu Rev Physiol. 2010;72:19–44. doi: 10.1146/annurev.physiol.010908.163111

41. Galluzzi L, Bravo-San Pedro JM, Kepp O, Kroemer G. Regulated cell death and adaptive stress responses. Cell Mol Life Sci. 2016;73:2405–2410. doi: 10.1007/s00018-016-2209-y

42. Galluzzi L, Leopold Soto A, Kumar S, Kroemer G. Caspases connect cell-death signaling to organismal homeostasis. Immunity. 2016;44:221–231. doi: 10.1016/j.immuni.2016.01.020

43. Weerasinghe P, Buja LM. Oncosis: an important non-apoptotic mode of cell death. Exp Mol Pathol. 2012;93:302–308. doi: 10.1016/j.yexmp.2012.09.018

44. Suzuki T, Franchi L, Toma C, Ashida H, Ogawa M, Yoshikawa Y, Mimuro H, Inohara N, Sasakawa C, Núñez G. Differential regulation of caspase-1 activation, pyroptosis, and autophagy via IAP and ASC in Shigella-infected macrophages. PLoS Pathog. 2007;3:e111. doi: 10.1371/journal.ppat.0030111

45. Chen X, He WT, Hu L, Li J, Fang Y, Wang X, Xu X, Wang Z, Huang K, Han J. Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis. Cell Res. 2016;26:1007–1020. doi: 10.1038/cr.2016.100

46. Aglietti RA, Decker EC. Recent insights into the molecular mechanisms underlying pyroptosis and gasdermin family functions. Trends Immunol. 2017;38:261–271. doi: 10.1016/j.it.2017.01.003

47. Abe J, Morrell C. Pyroptosis as a regulated form of necrosis: PLAXannein V→high caspase 1/low caspase 9 activity in cells is pyroptosis? Circ Res. 2012;110:1457–1460. doi: 10.1161/CIRCRESAHA.112.308699

48. Wright JA, Bryant CE. The killer protein gasdermin D. Cell Death Differ. 2016;23:1897–1898. doi: 10.1038/cdd.2016.100

49. Kayagaki N, Stowe IB, Lee BL, O’Rourke K, Anderson K, Warming S, Cuellar T, Haley B, Roose-Girma M, Phung QT, et al. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature. 2016;541:418–419. doi: 10.1038/nature21341

50. Man SM, Kanneganti TD. Gasdermin D: the long-awaited executioner of pyroptosis. Curr Biol. 2015;25:1183–1188. doi: 10.1016/j.cub.2015.12.014

51. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, Zhuya Y, Cai T, Wang F, Shao F. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature. 2015;526:660–666. doi: 10.1038/nature15514

52. Jorgensen I, Mao EA. Pyroptotic cell death defends against intracellular pathogens. Immunity. 2015;42:130–142. doi: 10.1016/j.immuni.2015.01.021

53. Willie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature. 1980;284:555–556. doi: 10.1038/284555a0

54. Shiokawa D, Maruta H, Tanuma S. Inhibitors of poly(ADP-ribose) polymerase suppress nuclear fragmentation and apoptotic-body formation during apoptosis in HL-60 cells. FEMS Lett. 1997;413:99–103. doi: 10.1016/S0168-6444(97)00087-9
49. Sager HB, Heidt T, Hulsmans M, Dutta P, Courties G, Sebas M, Wojtkiewicz GR, Tricot B, Iwamoto Y, Sun Y, et al. Targeting interleukin-1β reduces leukocyte production after acute myocardial infarction. Circulation 2015;132:1880–1890. doi: 10.1161/CIRCULATIONAHA.115.016160

50. Ackers-Johnson M, Li PY, Holmes AP, O’Brien SM, Pavlovic D, Foo RS. A simplified, langendorff-free method for concomitant isolation of viable cardiac myocytes and nonmyocytes from the adult mouse heart. Circ Res 2016;119:909–920. doi: 10.1161/CIRCRESAHA.116.309002

51. Xie L, Pi X, Wang Z, He J, Willis MS, Patterson C. Depletion of PHD3 protects heart from ischemia/reperfusion injury by inhibiting cardiomyocyte apoptosis. J Mol Cell Cardiol 2015;80:156–165. doi: 10.1016/j.yjmcc.2015.01.007

52. Jujo K, Ii M, Sekiguchi H, Kiyadoko E, Misener S, Tanaka T, Tongers J, Roncalli J, Renaud MA, Thorne T, et al. CXC-chemokine receptor 4 antagonist AMD3100 promotes cardiac functional recovery after ischemia/reperfusion injury via endothelial nitric oxide synthase-dependent mechanism. Circulation 2013;127:63–73. doi: 10.1161/CIRCULATIONAHA.112.099242

53. De Roeck L, Vandamme S, Eversart BR, Hoymans V, Haine S, Vandendriessche T, Bosmans J, Ronsyn MW, Miljoen H, Van Berendoncks A, et al. Adiponectin and ischemia-reperfusion injury in ST segment elevation myocardial infarction. Eur Heart J Acute Cardiovasc Care. 2016;5:71–76. doi: 10.1177/2048872615570770

54. Buller CE, Fu Y, Mahaffey KW, Todaro TG, Adams P, Westerhout CM, White HD, van’t Hof AW, Van de Werf FJ, Wagner GS, et al. ST-segment recovery and outcome after primary percutaneous coronary intervention for ST-elevation myocardial infarction: insights from the Assessment of Pexelizumab in Acute Myocardial Infarction (APEX-AMI) trial. Circulation. 2008;118:1335–1346. doi: 10.1161/CIRCULATIONAHA.108.767772

55. Michael LH, Entman ML, Hartley CJ, Youker KA, Zhu J, Hall SR, Hawkins HK, Berens K, Ballantyne CM. Myocardial ischemia and reperfusion: a murine model. Am J Physiol 1995;269:H2147–H2154. doi: 10.1152/ajpheart.1995.269.6.H2147