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Jie Yu et al., GPR43 alleviates myocardial I/R injury

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Abstract

Background: The purpose of this study is to investigate the regulatory role of G coupled-protein receptor 43 (GPR43) during myocardial ischemia/reperfusion (I/R) injury and to explore the relevant molecular mechanism.

Materials and methods: AC16 hypoxia/reoxygenation (H/R) model was established to simulate I/R injury *in vitro*. Gain- and loss-of-function experiments were conducted to regulate GPR43 or nesfatin1 expression. Cell viability and apoptosis was examined adopting CCK-8 and TUNEL assays. Commercial kits were applied for detecting ROS and inflammatory cytokines. Quantitative real-time PCR (qRT-PCR) and western blotting were conducted to measure the expression level of critical genes and proteins.

Results: GPR43 was downregulated in H/R-mediated AC16 cells. GPR43 overexpression or the GPR43 agonist greatly inhibited H/R-induced cell viability loss, cell apoptosis, and excessive production of ROS and pro-inflammatory cytokines in AC16 cardiomyocytes. Co-immunoprecipitation (Co-IP) assay identified an interaction between GPR43 and nesfatin1, and GPR43 could positively regulate

nesfatin1. In addition, the protective role of GPR43 against H/R injury was partly abolished upon nesfatin1 knockdown. Eventually, GPR43 could inhibit H/R-stimulated JNK/P38 MAPK signaling in AC16 cells, which was also hindered by nesfatin1 knockdown.

Conclusions: Our findings demonstrated the protective role of GPR43 against H/R-mediated cardiomyocytes injury through up-regulating nesfatin1, providing a novel target for the prevention and treatment of myocardial I/R injury.

Key words: hypoxia/reoxygenation, cardiomyocyte, GPR43, nesfatin1

INTRODUCTION

Acute myocardial infarction (AMI), also named as heart attack, is a main cause of cardiovascular disease-related deaths around the world(1). Timely myocardial reperfusion, such as thrombolysis and percutaneous coronary intervention, is crucial to reduce infarction size and improve clinical outcomes; however, the abrupt restoration of blood flow usually causes more serious damage and aggravates cardiomyocyte dysfunction, which is known as myocardial ischemia and reperfusion (I/R) injury(2, 3). Therefore, a better understanding of the molecular basis of myocardial I/R injury is urgently required to develop more effective strategies for relieving cardiomyocyte I/R damage.

G coupled-protein receptor 43 (GPR43), also known as free fatty acid receptor 2 (FFAR2), was originally cloned from white blood cells, and highly expressed in immune cells(4). GPR43 can bind short-chain fatty acids (SCFAs) produced by the microbial fermentation of carbohydrates to exhibit promising therapeutic potentials(5). In addition, GPR43 can activate intracellular calcium or cyclic adenosine monophosphate (cAMP) through coupling to the Gi/o or Gq signaling pathways, thereby transducing the downstream signaling cascades(6). Recent studies have demonstrated that GPR43 is widely distributed in immune cells, intestine tissues and adipose tissues, and is involved in multiple pathological conditions including

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cancer, obesity and inflammatory diseases(7-10). Recently, it is reported that the expression level of *GPR43* gene in peripheral blood of patients with AMI is remarkably downregulated compared to the healthy control, and the low *GPR43* level is considered as an independent risk factor for AMI, highlighting the importance of GPR43 for predicting AMI(11). Meanwhile, a latest document reveals that acetate exerts an inhibitory effect on cardiomyocyte contraction by activating GPR43, further indicating a potential involvement of GPR43 in cardiovascular function(12). Nonetheless, the specific regulatory role of GPR43 during the development of AMI, especially cardiomyocyte I/R damage, is still ambiguous.

In the current work, we aimed to investigate the functional role and the relevant mechanism of GPR43 in myocardial I/R injury, which will provide novel strategies for the attenuation of cardiomyocyte I/R damage.

MATERIALS AND METHODS

Cell culture and induction

Human cardiomyocytes (AC16) cells were purchased from BeNa Culture Collection (Beijing, China), and were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 ℃ in the absence of 5% CO_2.

AC16 hypoxia/reoxygenation (H/R) model was established to simulate I/R injury *in vitro* as previously described(13). AC16 cells were exposed to a glucose- and serum-free medium in a hypoxic incubator with 5% CO_2 and 95% N_2 at 37 ℃ for hypoxia. 10 h later, AC16 cells were reoxygenated by incubation with the fresh DMEM medium and transferring into a normoxic incubator with 5% CO_2 and 95% air for 8 h.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from AC16 cells adopting the Trizol reagent (Invitrogen, Carlsbad, CA, USA). A Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA) was utilized for the RNA quantification and purity

detection. 1 μg of RNA was then reverse-transcribed into complementary DNA (cDNA), followed by qRT-PCR adopting SYBR Green (Applied Biosystems, Carlsbad, CA). Each gene expression level was calculated using $2^{-\triangle\triangle Ct}$ method and normalized to β-actin.

Western blotting

Total protein was extracted from AC16 cells adopting RIPA lysis buffer (Beyotime, China). After the determination of protein concentration using BCA assay, the equal amounts of protein were denatured by boiling for 5 min, subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After being blocked with 5% skimmed milk for 1 h, the membranes were probed with primary antibodies at 4 ℃ overnight, followed by the incubation with the horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. Eventually, the blots were visualized using Pierce ECL Western Blotting Substrate and quantified using ImageJ software (National Institutes of Health, Bethesda, MD). GAPDH served as the internal control.

Cell transfection

Open reading frames of *GPR43* gene were amplified and inserted into pcDNA3.1 plasmid to generate a pcDNA3.1-GPR43 overexpression vector (Ov-GPR43), and the scramble pcDNA3.1 plasmid served as the negative control (Ov-NC). Meanwhile, the short hairpin RNA targeting Nesfatin (shRNA-Nesfatin-1 and shRNA-Nesfatin-2), as well as its negative control (shRNA-NC) were obtained from GenePharma (Shanghai, China). Transfections with above plasmids, respectively, were conducted using the Lipofectamine 3000 RNAiMax reagent (Invitrogen) strictly in line with the protocol of the manufacturer. 48 h post transfection, the transfection efficiency was verified via qRT-PCR or western blotting.

Cell viability assay

Cell viability was detected employing cell counting kit-8 (CCK-8) assay. Briefly, AC16 cells were cultivated into 96-well plates overnight. After indicated treatment, 10 µl of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well and the cells were incubated at 37 ℃ for another 2 h. Eventually, the absorbance at 450 nm of each well was measured under a microplate reader (BioTek, Nanjing, China).

Reactive oxygen species (ROS) assay

The intracellular ROS level of AC16 cells was detected by a fluorescent probe 2',7'-Dichlorodi-hydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St. Louis, USA) in accordance with the manufacturer's procedure. Cells were incubated with 10 µM DCFH for 1 h, and then the fluorescence images were captured by a fluorescence microscope (Olympus, Tokyo, Japan).

Detection of inflammatory cytokines

AC16 cells were collected and centrifugated for the harvest of supernatant. Thereafter, the inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β in the supernatant of culture medium was detected in strict accordance with the instruction of corresponding kits (R&D Systems, USA).

TUNEL assay

Cell apoptosis was evaluated by TUNEL assay. In brief, AC16 cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 at room temperature. Subsequently, cells were incubated with TUNEL reaction mixture (Roche, Basel, Switzerland) at 37 ℃ for 1 h away from the light in line with the protocol of manufacturer. The apoptotic cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Co-immunoprecipitation (Co-IP) assay

The total proteins of AC16 cells were extracted adopting RIPA lysis buffer

(Beyotime, China) and then incubated with IP-indicated antibodies and IgG, and untreated proteins served as an input control. The mixture was incubated with protein A/G agarose beads (Santa Cruz Biotechnology, USA). The immunoprecipitated protein complex was boiled and denatured. Following centrifugation, the supernatant was subjected to western blotting analysis.

Statistical analysis

Data were analyzed employing GraphPad Prims version 8.0 and presented as mean ± standard deviation (SD). One-way ANOVA with Tukey's post hoc test was employed for comparisons among groups and Student's t-test was used for comparison within two groups. A p value less than 0.05 was considered statistically significant.

RESULTS

GPR43 is downregulated in AC16 H/R model and GPR43 overexpression inhibits H/R-induced apoptosis in AC16 cardiomyocytes

To examine the role of GPR43 during cardiomyocyte I/R damage, we constructed the H/R cell model using human cardiomyocytes AC16. It was clearly observed from Figure 1A-B that both the mRNA level and protein expression of GPR43 was declined following H/R procedure in AC16 cells. Thus, a gain-of-function experiment was performed to assess the regulatory role of GPR43 in AC16 H/R model. As shown in Figure 2A-B, the GPR43 was greatly overexpressed after transfection with Ov-GPR43. Subsequently, the GPR43-overexpressing AC16 cells were exposed to H/R procedure, accompanied with that the un-transfected AC16 cells were exposed to H/R procedure in the presence of the specific GPR43 agonist (S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenyl thiazole-2-yl) butanamide (PA; 0.5 μM). The data from CCK-8 assay revealed that H/R caused abundant cell viability loss in AC16 cells, which was greatly attenuated by Ov-GPR43 transfection or PA (Figure 2C). Meanwhile, as exhibited in Figure 2D, the TUNEL-positive cells were greatly elevated in H/R-

exposed AC16 cells, which was then hindered by Ov-GPR43 transfection or PA, suggesting that GPR43 could alleviate H/R-induced apoptosis in AC16 cells, which was further evidenced by the inhibitory effects of Ov-GPR43 transfection or PA on the downregulated Bcl-2 and upregulated Bax and cleaved caspase3 in H/R-induced AC16 cells (Figure 2E).

GPR43 counteracts H/R-induced oxidative stress and inflammation in AC16 cardiomyocytes

Next, we investigated the impacts of GPR43 on ROS and crucial cytokines involved in cardiomyocyte I/R damage. The results from Figure 3A presented that H/R stimulation greatly elevated ROS level in AC16 cells, which was then abolished by Ov-GPR43 transfection or PA, indicating that GPR43 could hinder H/R-mediated oxidative stress in AC16 cells. In addition, the concentrations of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α were remarkably increased upon H/R exposure, and Ov-GPR43 transfection or PA treatment greatly hindered these changes (Figure 3B), which is consistent with the following qRT-PCR assay examining the mRNA level of IL-6, IL-1β and TNF-α in each group (Figure 3C). In addition, it was also found that H/R stimulation hugely increased the protein level of p-p65 and p-IKβα of AC16 cells, while Ov-GPR43 transfection or PA treatment partially reversed these alternations (Figure 3D).

GPR43 interacts with nesfatin1 and positively regulates nesfatin1

Next, we continued to elucidate the regulatory mechanism of GPR43 against H/R-mediated AC16 cardiomyocytes injury. It is found from BioGRID website (https://thebiogrid.org/) that GPR43 may be bound to nesfatin1. Thus, we examined nesfatin1 expression underlying GPR43 regulation. According to the qRT-PCR and western blotting assays, it was confirmed that the mRNA level and protein expression of nesfatin1 was significantly downregulated in H/R-mediated AC16 cells, which was then restored following Ov-GPR43 transfection or PA treatment (Figure 4A-B).

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Furthermore, the subsequent Co-IP assay verified that GPR43 and nesfatin1 did interact with each other in AC16 cells (Figure 4C-D).

Nesfatin1 knockdown diminishes the protective role of GPR43 against H/R-induced apoptosis, oxidative stress and inflammation in AC16 cardiomyocytes

To confirm whether nesfatin1 is responsible for the regulation of GPR43 in H/R-mediated AC16 cells, AC16 cells were transfected with shRNA-Nesfatin1-1/2 or shRNA-NC. Compared to the shRNA-NC group, shRNA-Nesfatin1-1/2 led to a great reduction of nesfatin1 at both mRNA level and protein expression (Figure 5A-B). Attributed to a higher knockdown efficacy, shRNA-Nesfatin1-1 was used in the following experiments. Thereafter, the results from CCK-8 assay and TUNEL assay revealed that the inhibitory effects of PA on H/R-induced cell viability loss and cell apoptosis in AC16 cells were partly weakened by nesfatin1 knockdown (Figure 5C-D), which was further proved by the subsequent downregulated Bcl-2 expression and the upregulated Bax and cleaved caspase3 expression in H/R+PA+shRNA-Nesfatin1 group in comparison to H/R+PA+shRNA-NC group (Figure 5E). Moreover, nesfatin1 knockdown also greatly weakened the inhibitory effects of PA on H/R-mediated high ROS level and pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) production (Figure 6A-C). As expected, the reduced protein expression of p-p65 and p-IKβα in PA-treated AC16 cells partly rose again following nesfatin1 knockdown (Figure 6D). The above findings demonstrated that the inhibitory effects of PA on H/R-mediated cell viability loss, cell apoptosis, oxidative stress and inflammation were partly weakened by nesfatin1 knockdown, suggesting that the protective role of PA against H/R-mediated cardiomyocytes injury was achieved partly through upregulating nesfatin1.

GPR43/nesfatin1 modulates JNK/P38 MAPK signaling in H/R-exposed AC16 cardiomyocytes

Finally, we also evaluated the JNK/P38 MAPK signaling underlying the

regulation of GPR43/nesfatin1 in H/R-exposed AC16 cardiomyocytes. The results from western blotting assay revealed that the protein expression of p-JNK and p-p38 was hugely increased following H/R exposure, while PA treatment remarkably repressed this elevation, suggesting that GPR43 could inhibit the activation of JNK/P38 MAPK signaling induced by H/R injury. In addition, the repressed JNK/P38 MAPK signaling caused by PA was partly restored upon nesfatin1 knockdown, demonstrating a close involvement of GPR43/nesfatin1 axis in regulating H/R-stimulated JNK/P38 MAPK signaling in AC16 cells (Figure 7).

DISCUSSION

I/R injury has been regarded as one of the most common pathophysiological features of AMI, and has been developed to be one of the severe risks contributing to the high mortality of AMI(14, 15). Mitigating I/R-induced cardiomyocyte damage and consequently preserving cardiomyocyte function are crucial for improving the treatment of AMI. In this study, we for the first time found that GPR43 was downregulated during H/R-mediated cardiomyocyte injury, and then demonstrated a novel cardioprotective role of GPR43 in cardiomyocytes following H/R. We believe our findings provide evidence of the potential of GPR43 as a treatment target against myocardial I/R injury.

In recent years, accumulating documents have evidenced multiple pathophysiological processes during cardiomyocyte I/R injury, including overproduction of pro-inflammatory cytokines, excessive generation of ROS, calcium overload, energy metabolism and cardiomyocyte cell apoptosis(16-18). Under physiological condition, ROS is produced as a result of normal cellular metabolism processes; however, the abnormal elevated ROS occurs in both ischemia and reperfusion periods(19). During reperfusion, ROS can activate JNK/P38 MAPK signaling, followed by the dissociation of the NF-κB from its inhibitor IκB to upregulate activated NF-κB(20). NF-κB is a well-recognized signaling which triggers the production of inflammatory cytokines such as IL-6, IL-1β and TNF-α(21, 22).

Currently, much attention has been paid on these aspects to seek for effective targets or drugs to alleviate myocardial I/R injury. For example, Nie C et al. pointed out that hydrogen gas inhalation was beneficial to mitigate myocardial I/R injury through inhibiting oxidative stress and reducing the release of IL-1β(23); Zhai M et al. suggested melatonin as a potential drug for ameliorating myocardial I/R injury attributed to its potent anti-apoptosis and anti-oxidative stress properties(24); Guanxinning Injection, used clinically to treat cardiac injury, was demonstrated to improve I/R-induced myocardial injury through inhibiting NF-κB inflammation signaling pathway(25). Therefore, targeting minishing oxidative stress, inflammation and apoptosis is practicable to ameliorate myocardial I/R injury.

In terms of GPR43, it is discovered that GPR43 overexpression or the GPR43 agonist inhibited high glucose-induced oxidative stress and NF-κB activation, acting as an important mediator for preventing diabetic nephropathy(26). Park BO et al. revealed several novel GPR43 agonists exerted strong anti-inflammatory potential in attenuating colitis via downregulating NF-κB activity(27). A latest report also demonstrated a protective role of GPR43 in mediating lung injury through inhibiting inflammation and apoptosis via JNK pathway(28). Hence, GPR43 itself possesses outstanding activities against oxidative stress, inflammation and cell apoptosis, which may account for its widely potential application for the prevention and treatment of multiple diseases. As expected, our findings illustrated that no matter GPR43 overexpression or the GPR43 agonist could remarkably inhibit H/R-mediated oxidative stress, inflammation and apoptosis in AC16 cardiomyocytes, accompanied with the downregulated NF-κB and JNK/P38 MAPK signaling activities.

Moreover, to further explore the molecular mechanism of the protective role of GPR43 against H/R-induced cardiomyocytes injury, we discovered that GPR43 might interact with nesfatin1 and positively regulate nesfatin1 in AC16 cardiomyocytes. Nesfatin1, also named as nucleobindin-2 (NUCB2), is an 82-amino acid peptide initially discovered by Oh-1 and his colleagues in 2006(29). The subsequent development gradually uncovered the pleiotropic effects of nesfatin1, and up to date,

nesfatin1 has been recognized to exert numerous biological effects, including maintaining the feeding balance and glucose/lipid homeostasis, affecting energy metabolism and psychological disorders, and modulating gastrointestinal and cardiovascular functions(30-34). It is notable that nesfatin-1 exhibits anti-inflammatory, and anti-apoptotic and antioxidant properties(35). In addition, nesfatin1 has been proved to attenuate myocardial I/R injury by targeting inflammation, oxidative stress and apoptosis, serving as a well candidate to treat AMI(36, 37). In the present study, we proved that nesfatin1 knockdown partly weakened the inhibitory effects of GPR43 on H/R-mediated inflammation, oxidative stress and apoptosis in AC16 cardiomyocytes. Given the interaction between GPR43 and nesfatin1, as well as their positive correlation, the protective role of GPR43 against H/R injury may be partly dependent on upregulating nesfatin1.

CONCLUSIONS

In summary, our findings for the first time demonstrated the protective role of GPR43 against H/R-mediated cardiomyocytes injury via inhibiting oxidative stress, inflammation and cell apoptosis. In detail, this protection action of GPR43 is achieved partly through up-regulating nesfatin1, which is also linked to the modulation of JNK/P38 MAPK signaling. Overall, this study provides a novel target for the prevention and treatment of myocardial I/R injury.

Conflict of interest: None declared

REFERENCES

1. Yousuf T, Nakhle A, Rawal H, Harrison D, Maini R and Irimpen A: Natural disasters and acute myocardial infarction. Prog Cardiovasc Dis 63: 510-517, 2020.
2. Chamberlain JJ, Johnson EL, Leal S, Rhinehart AS, Shubrook JH and Peterson L: Cardiovascular Disease and Risk Management: Review of the American Diabetes Association Standards of Medical Care in Diabetes 2018. Ann Intern Med 168: 640-650, 2018.
3. Tong X, Chen J, Liu W, Liang H and Zhu H: LncRNA LSINCT5/miR-222 regulates myocardial ischemia-reperfusion injury through PI3K/AKT pathway. J Thromb Thrombolysis 52: 720-729, 2021.
4. Senga T, Iwamoto S, Yoshida T, *et al*: LSSIG is a novel murine leukocyte-specific GPCR that is

induced by the activation of STAT3. Blood 101: 1185-1187, 2003.

5.   Bindels LB, Dewulf EM and Delzenne NM: GPR43/FFA2: physiopathological relevance and therapeutic prospects. Trends Pharmacol Sci 34: 226-232, 2013.

6.   Lymperopoulos A, Suster MS and Borges JI: Short-Chain Fatty Acid Receptors and Cardiovascular Function. Int J Mol Sci 23: 2022.

7.   Hu J, Kyrou I, Tan BK, *et al*: Short-Chain Fatty Acid Acetate Stimulates Adipogenesis and Mitochondrial Biogenesis via GPR43 in Brown Adipocytes. Endocrinology 157: 1881-1894, 2016.

8.   McKenzie CI, Mackay CR and Macia L: GPR43 - A Prototypic Metabolite Sensor Linking Metabolic and Inflammatory Diseases. Trends Endocrinol Metab 26: 511-512, 2015.

9.   Zhang C, Chang J, Wu W, *et al*: Activation of GPR43 suppresses TNF-alpha-induced inflammatory response in human fibroblast-like synoviocytes. Arch Biochem Biophys 684: 108297, 2020.

10.   Lu X, Qiao S, Peng C, *et al*: Bornlisy Attenuates Colitis-Associated Colorectal Cancer via Inhibiting GPR43-Mediated Glycolysis. Front Nutr 8: 706382, 2021.

11.   Ruan J, Meng H, Wang X, Chen W, Tian X and Meng F: Low Expression of FFAR2 in Peripheral White Blood Cells May Be a Genetic Marker for Early Diagnosis of Acute Myocardial Infarction. Cardiol Res Pract 2020: 3108124, 2020.

12.   Jiang X, Zhang Y, Zhang H, *et al*: Acetate suppresses myocardial contraction via the short-chain fatty acid receptor GPR43. Front Physiol 13: 1111156, 2022.

13.   Huang G, Lu X, Duan Z, *et al*: PCSK9 Knockdown Can Improve Myocardial Ischemia/Reperfusion Injury by Inhibiting Autophagy. Cardiovasc Toxicol 22: 951-961, 2022.

14.   Turer AT and Hill JA: Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. Am J Cardiol 106: 360-368, 2010.

15.   Maneechote C, Palee S, Chattipakorn SC and Chattipakorn N: Roles of mitochondrial dynamics modulators in cardiac ischaemia/reperfusion injury. J Cell Mol Med 21: 2643-2653, 2017.

16.   Li SY, Li ZX, He ZG, *et al*: Quantitative proteomics reveal the alterations in the spinal cord after myocardial ischemia-reperfusion injury in rats. Int J Mol Med 44: 1877-1887, 2019.

17.   Bagheri F, Khori V, Alizadeh AM, Khalighfard S, Khodayari S and Khodayari H: Reactive oxygen species-mediated cardiac-reperfusion injury: Mechanisms and therapies. Life Sci 165: 43-55, 2016.

18.   White CR, Giordano S and Anantharamaiah GM: High-density lipoprotein, mitochondrial dysfunction and cell survival mechanisms. Chem Phys Lipids 199: 161-169, 2016.

19.   Xiang M, Lu Y, Xin L, *et al*: Role of Oxidative Stress in Reperfusion following Myocardial Ischemia and Its Treatments. Oxid Med Cell Longev 2021: 6614009, 2021.

20.   Zhao ZQ: Oxidative stress-elicited myocardial apoptosis during reperfusion. Curr Opin Pharmacol 4: 159-165, 2004.

21.   Yang W, Lai Q, Zhang L, *et al*: Mechanisms dissection of the combination GRS derived from ShengMai preparations for the treatment of myocardial ischemia/reperfusion injury. J Ethnopharmacol 264: 113381, 2021.

22.   Wang R, Wang M, Zhou J, *et al*: Shuxuening injection protects against myocardial ischemia-reperfusion injury through reducing oxidative stress, inflammation and thrombosis. Ann Transl Med 7: 562, 2019.

23.   Nie C, Ding X, A R, *et al*: Hydrogen gas inhalation alleviates myocardial ischemia-reperfusion injury by the inhibition of oxidative stress and NLRP3-mediated pyroptosis in rats. Life Sci 272:

119248, 2021.

24. Zhai M, Li B, Duan W, *et al*: Melatonin ameliorates myocardial ischemia reperfusion injury through SIRT3-dependent regulation of oxidative stress and apoptosis. J Pineal Res 63: 2017.

25. Xiao G, Liu J, Wang H, *et al*: CXCR1 and its downstream NF-kappaB inflammation signaling pathway as a key target of Guanxinning injection for myocardial ischemia/reperfusion injury. Front Immunol 13: 1007341, 2022.

26. Huang W, Man Y, Gao C, *et al*: Short-Chain Fatty Acids Ameliorate Diabetic Nephropathy via GPR43-Mediated Inhibition of Oxidative Stress and NF-kappaB Signaling. Oxid Med Cell Longev 2020: 4074832, 2020.

27. Park BO, Kang JS, Paudel S, *et al*: Novel GPR43 Agonists Exert an Anti-Inflammatory Effect in a Colitis Model. Biomol Ther (Seoul) 30: 48-54, 2022.

28. Xu Q, Xu J and Wu Y: Regulation of inflammation and apoptosis by GPR43 via JNK/ELK1 in acute lung injury. Inflamm Res 71: 603-614, 2022.

29. Oh IS, Shimizu H, Satoh T, *et al*: Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 443: 709-712, 2006.

30. Li Z, Gao L, Tang H, *et al*: Peripheral effects of nesfatin-1 on glucose homeostasis. PLoS One 8: e71513, 2013.

31. Blanco AM, Velasco C, Bertucci JI, Soengas JL and Unniappan S: Nesfatin-1 Regulates Feeding, Glucosensing and Lipid Metabolism in Rainbow Trout. Front Endocrinol (Lausanne) 9: 484, 2018.

32. Goebel-Stengel M and Stengel A: Role of Brain NUCB2/nesfatin-1 in the Stress-induced Modulation of Gastrointestinal Functions. Curr Neuropharmacol 14: 882-891, 2016.

33. Angelone T, Rocca C and Pasqua T: Nesfatin-1 in cardiovascular orchestration: From bench to bedside. Pharmacol Res 156: 104766, 2020.

34. Xiao MM, Li JB, Jiang LL, Shao H and Wang BL: Plasma nesfatin-1 level is associated with severity of depression in Chinese depressive patients. BMC Psychiatry 18: 88, 2018.

35. Xu Y and Chen F: Antioxidant, Anti-Inflammatory and Anti-Apoptotic Activities of Nesfatin-1: A Review. J Inflamm Res 13: 607-617, 2020.

36. Su RY, Geng XY, Yang Y and Yin HS: Nesfatin-1 inhibits myocardial ischaemia/reperfusion injury through activating Akt/ERK pathway-dependent attenuation of endoplasmic reticulum stress. J Cell Mol Med 25: 5050-5059, 2021.

37. Naseroleslami M, Sharifi M, Rakhshan K, Mokhtari B and Aboutaleb N: Nesfatin-1 attenuates injury in a rat model of myocardial infarction by targeting autophagy, inflammation, and apoptosis. Arch Physiol Biochem 129: 122-130, 2023.

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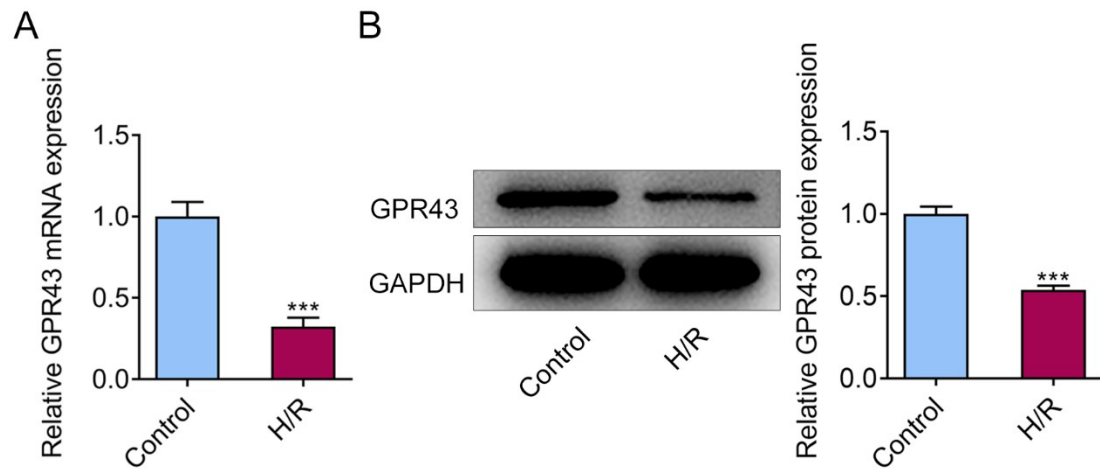
Figure 1. GPR43 is downregulated in AC16 H/R model. The H/R cell model was constructed using human cardiomyocytes AC16 to simulate cardiomyocyte I/R damage. The (A) mRNA level and (B) protein expression of GPR43 was detected by qRT-PCR and western blotting, respectively. ***$p<0.001$.
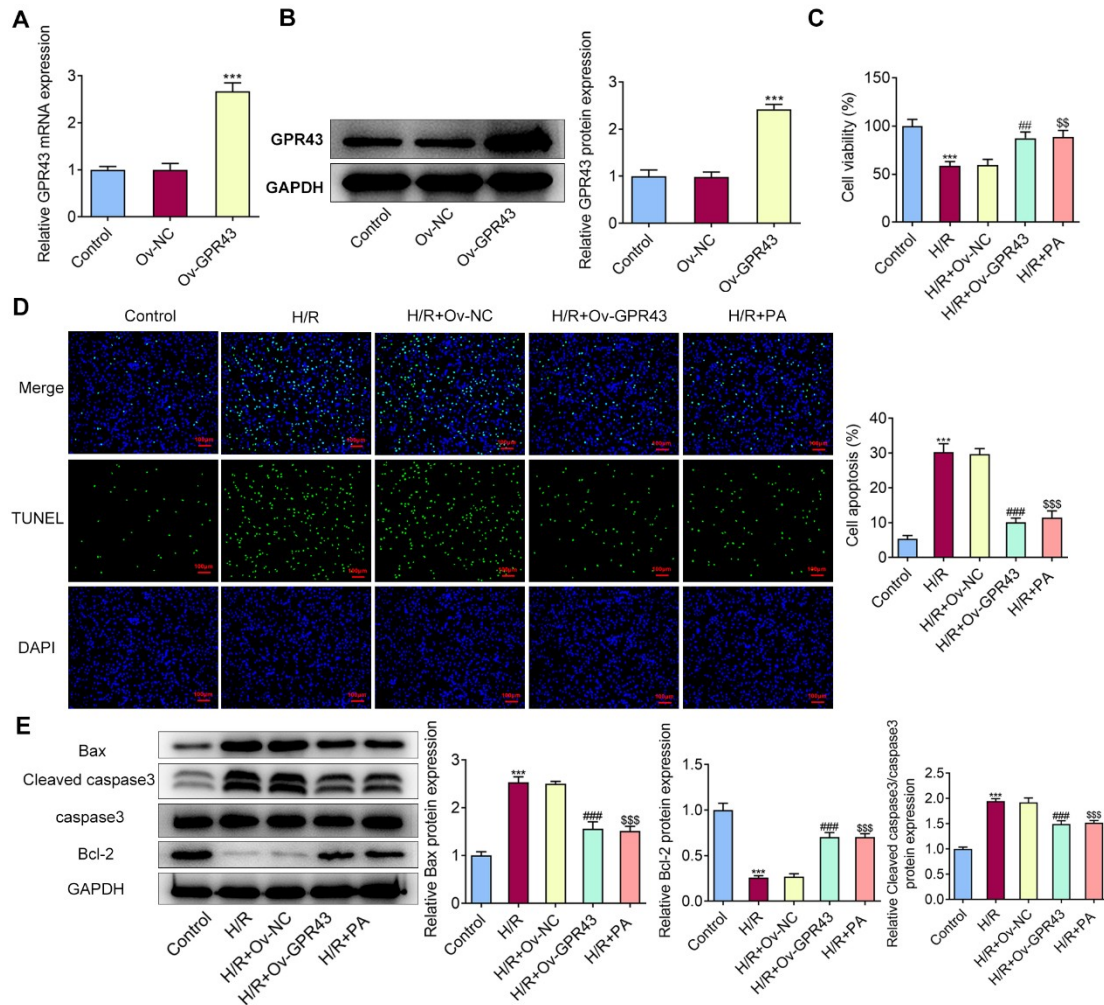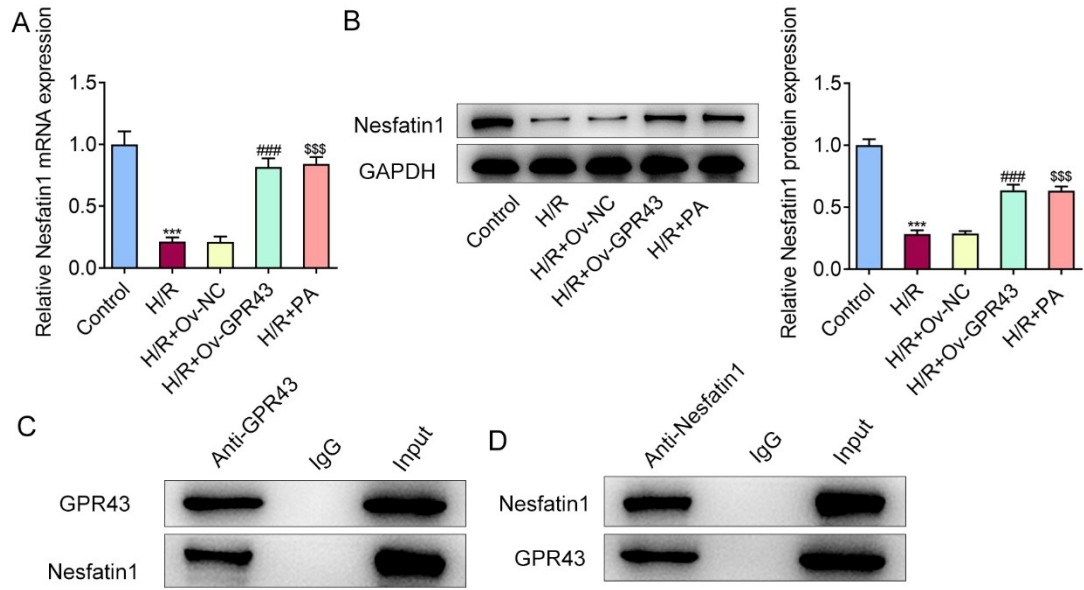
Figure 2. GPR43 overexpression inhibits H/R-induced apoptosis in AC16 cardiomyocytes. AC16 cells were transfected with Ov-NC or Ov-GPR43, and the (A) mRNA level and (B) protein expression of GPR43 was detected by qRT-PCR and western blotting, respectively. ***p<0.001 vs Ov-NC; (C) The GPR43-overexpressing AC16 cells were exposed to H/R procedure, accompanied with that the un-transfected AC16 cells were exposed to H/R procedure in the presence of the specific GPR43 agonist PA (0.5 μM). CCK-8 assay was performed to examine cell viability. (D) TUNEL assay was conducted to analyze cell apoptosis. (E) The protein expression of Bcl-2, Bax, cleaved caspase3 and caspase3 was detected by western blotting. ***p<0.001 vs Control; ##p<0.01 and ###p<0.001 vs H/R+Ov-NC; $$p<0.01 and $$$p<0.001 vs H/R.
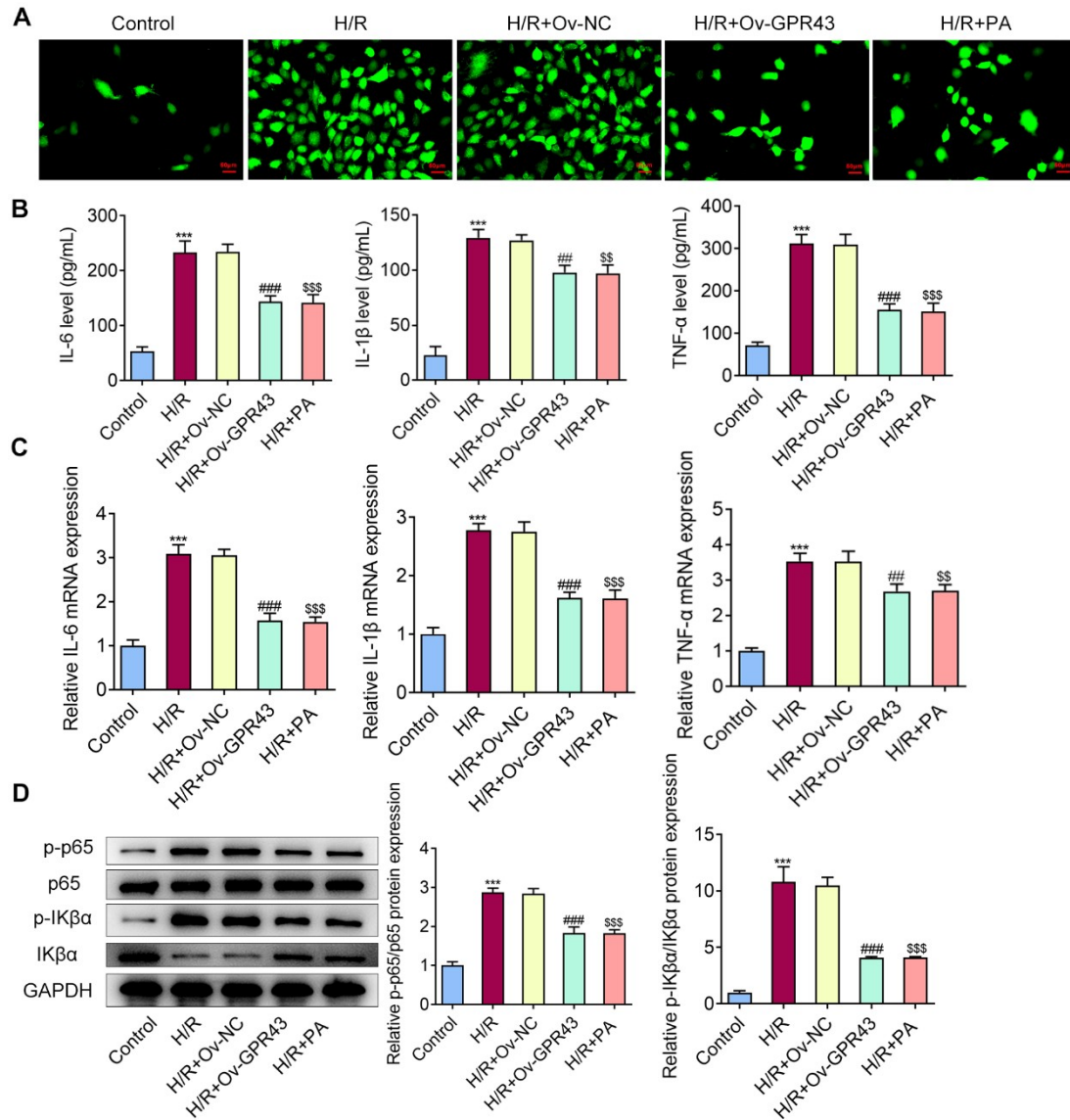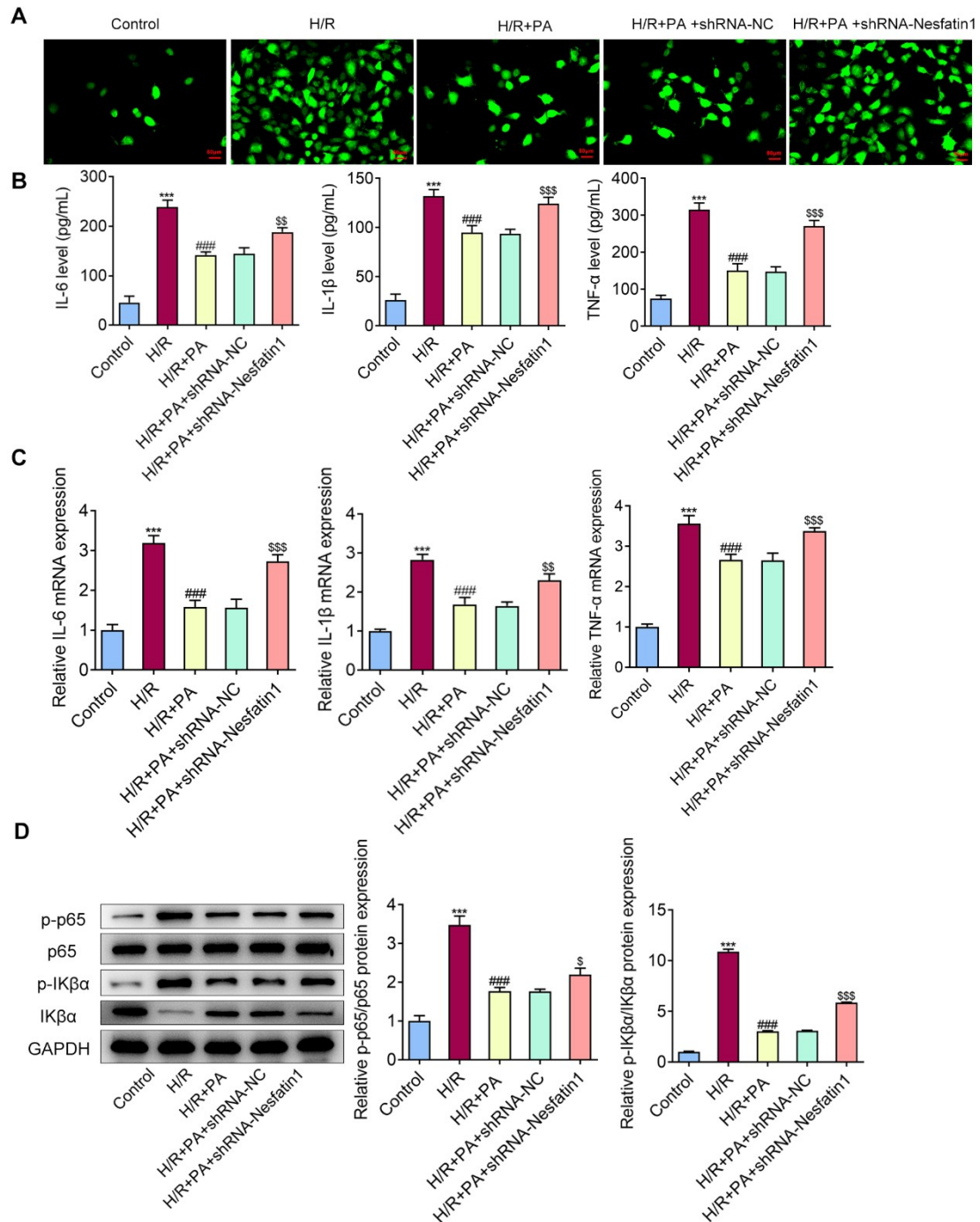
Figure 3. GPR43 counteracts H/R-induced oxidative stress and inflammation in AC16 cardiomyocytes. (A) ROS level in AC16 cells were examined using DCFH method. (B) The concentrations of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α were detected via ELISA kits. (C) The mRNA level of IL-6, IL-1β and TNF-α were detected via qRT-PCR assay. (D) The protein expression of p-p65, p65, p-IKβα and IKβα was detected by western blotting. ***$p<0.001$ vs Control; ##$p<0.01$ and ###$p<0.001$ vs H/R+Ov-NC; $$$p<0.01$ and $$$$p<0.001$ vs H/R.

Figure 4. GPR43 interacts with nesfatin1 and positively regulates nesfatin1. The (A) mRNA level and (B) protein expression of GPR43 was detected by qRT-PCR and western blotting, respectively. ***p<0.001 vs Control; ###p<0.001 vs H/R+Ov-NC; $$$p<0.001 vs H/R. (C-D) Co-IP assay was performed, followed by western blotting to confirm the interaction between GPR43 and nesfatin1.
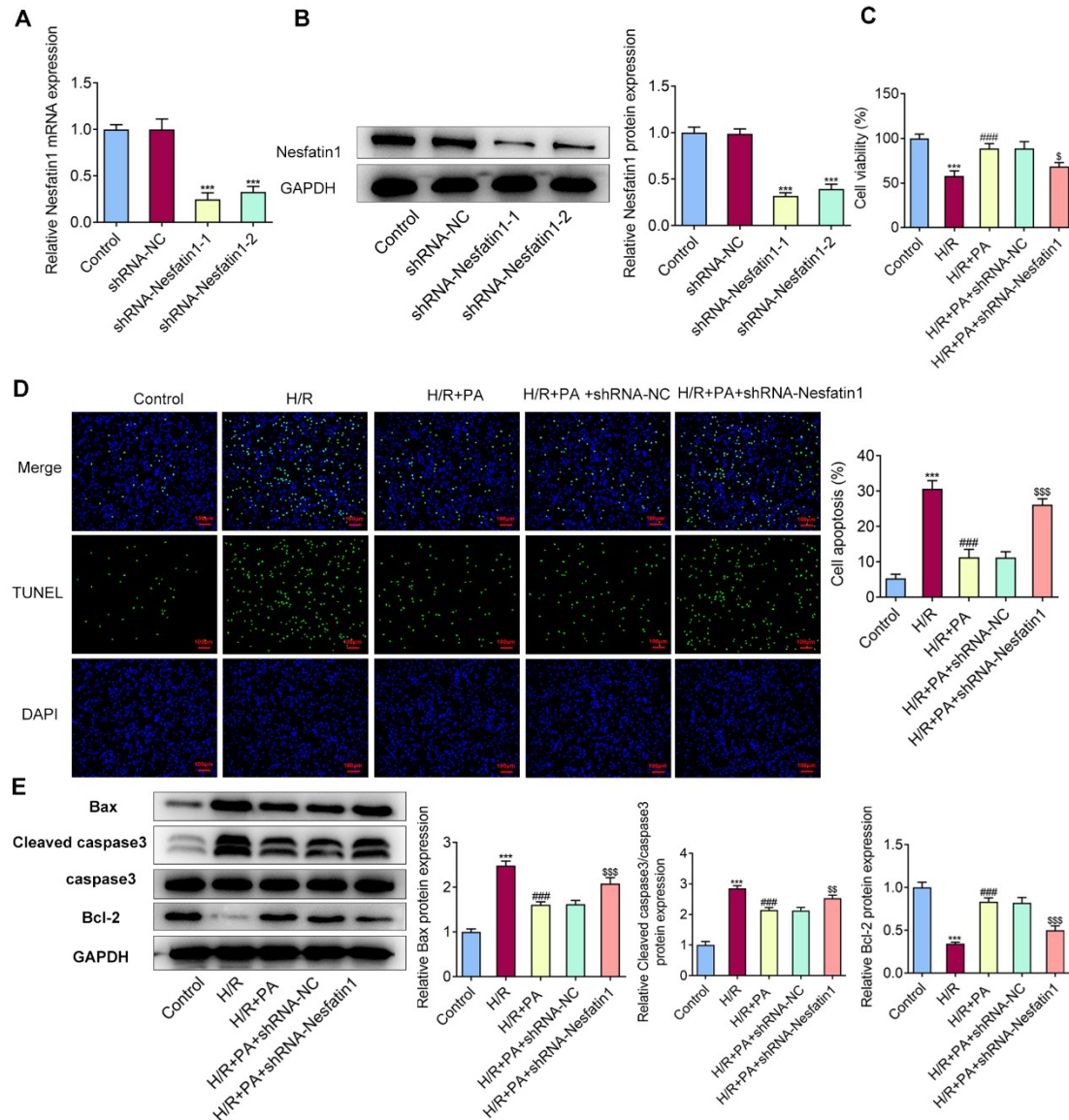
Figure 5. Nesfatin1 knockdown diminishes the protective role of GPR43 against H/R-induced apoptosis in AC16 cardiomyocytes. AC16 cells were transfected with shRNA-Nesfatin1-1/2 or shRNA-NC. The (A) mRNA level and (B) protein expression of nesfatin1 was detected by qRT-PCR and western blotting, respectively. ***p<0.001 vs shRNA-NC; (C) CCK-8 assay was performed to examine cell viability. (D) TUNEL assay was conducted to analyze cell apoptosis. (E) The protein expression of Bcl-2, Bax, cleaved caspase3 and caspase3 was detected by western blotting. ***p<0.001 vs Control; ###p<0.001 vs H/R; $p<0.05, $$p<0.01 and $$$p<0.001 vs H/R+PA+shRNA-NC.

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Figure 6. Nesfatin1 knockdown diminishes the protective role of GPR43 against H/R-induced oxidative stress and inflammation in AC16 cardiomyocytes. (A) ROS level in AC16 cells were examined using DCFH method. (B) The concentrations of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α were detected via ELISA kits. (C) The mRNA level of IL-6, IL-1β and TNF-α were detected via qRT-PCR assay. (D) The protein expression of p-p65, p65, p-IKβα and IKβα was detected by western blotting. ***p<0.001 vs Control; ###p<0.001 vs H/R; $p<0.05, $$p<0.01 and $$$p<0.001 vs H/R+PA+shRNA-NC.
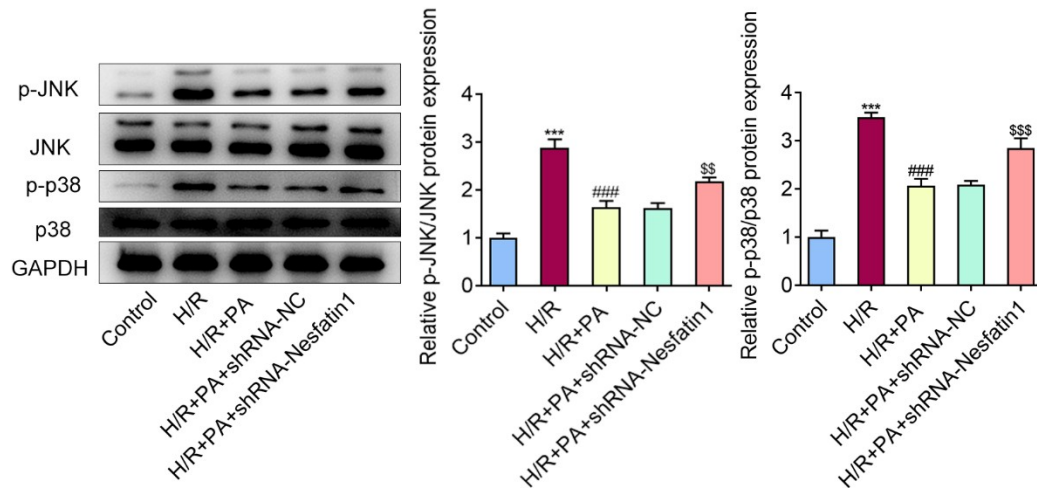
Figure 7. GPR43/nesfatin1 modulates JNK/P38 MAPK signaling in H/R-exposed AC16 cardiomyocytes. The protein expression of p-JNK, JNK, p-p38, p38 was detected by western blotting. ***p<0.001 vs Control; ###p<0.001 vs H/R; $$p<0.01 and $$$p<0.001 vs H/R+PA+shRNA-NC.