

Sodium valproate inhibited apoptosis in lethally scalded rat cardiomyocytes by regulating hypoxia-inducible factor-1 α expression

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Abstract: This study assessed the inhibitory effect of sodium valproate (VPA) on apoptosis of cardiomyocytes in lethally scalded rats. The model of a 50% total body surface area (TBSA) third-degree full-thickness scald was produced, 48 male SD rats were randomly divided into three groups (n = 16), the sham group and the scald group were given an intraperitoneal injection of 0.25ml of saline, the scald +VPA group was given an intraperitoneal injection of VPA (300 mg/kg) after scalded, Each group was subdivided into two subgroups (n=8) according to the two observation time points of 3h and 6h after scald. Apoptotic cardiomyocytes were observed, and myocardial tissue levels of nitric oxide (NO), cysteine protease-3 (caspase-3) activity, hypoxia-inducible factor-1 α (HIF-1 α), inducible nitric oxide synthase (iNOS), BCL2/adenovirus E1B interacting protein 3 (BNIP3) and caspase-3 protein were measured. Compared with sham scald group, severe scald elevated CK-MB, cardiomyocyte apoptosis rate, caspase-3 activity and protein levels, NO content, and HIF-1 α signalling pathway proteins; whereas VPA decreased CK-MB, cardiomyocyte apoptosis rate and inhibited HIF-1 α signalling pathway protein expression. In conclusion, these results suggested that VPA inhibited early cardiomyocyte apoptosis and attenuated myocardial injury in lethally scalded rats, which may be related to the regulation of the HIF-1 α signalling pathway.

Keywords: Scald; sodium valproate; cardiomyocyte; apoptosis; hypoxia-inducible factor-1 α

INTRODUCTION

After severe burns, circulating blood volume is dramatically reduced, blood perfusion to tissues and all important organs is insufficient, and tissue ischemia and hypoxia cause damage to cardiac parenchymal cells (Terengova and Tousek, 2017 ;Guillory *et al.*, 2016; Kazams, *et al.*, 2022). Early onset of myocardial injury after severe burns is closely related to apoptosis of cardiomyocytes (Zhang *et al.*, 2008), which is accompanied by the generation of inflammatory factors, the production of oxygen free radicals, activation of the PI3K/Akt pathway (Cao *et al.*, 2011) and an increase in the expression of caspase-3 (Ye *et al.*, 2022), which further induces apoptosis of cardiac myocytes. Under the conditions of ischemia and hypoxia in myocardial tissue, the level of HIF-1 α protein in myocardial cells increases and activates the downstream target genes related to hypoxia, resulting in increased vascular permeability, decreased myocardial contractile function, impaired energy metabolism, induced apoptosis of myocardial cells, and impaired cardiac function (Zheng *et al.*, 2021; Zhang *et al.*, 2018; Datta *et al.*, 2021).

Sodium valproate (VPA), a histone deacetylase inhibitor, has traditionally been used in the treatment of epilepsy and has been shown to be a safe and effective treatment

(Wang *et al.*, 2022). Previous studies have found that VPA can increase the early survival rate of severe burns, improve myocardial enzymatic indices and histological damage in scalded shock rats, improve pulmonary capillary permeability (Luo *et al.*, 2014; Hu *et al.*, 2012), reduce the need for resuscitation of pigs in hemorrhagic shock, and improve hemodynamic and laboratory indices, from which we can deduce that VPA improves cardiac function in hypovolemic shock animal models (Williams *et al.*, 2019). The heart is an important organ for maintaining the basic life of the organism and one of the most vulnerable organs after severe burns (Zhang *et al.*, 2023), and myocardial injury is associated with increased apoptosis of cardiomyocytes.

We hypothesised that VPA inhibits apoptosis of cardiomyocytes in the early stage of severe scald injury and improves myocardial injury. To test this hypothesis, we evaluated the effect of VPA on apoptosis in lethal scalded cardiomyocytes and changes in the expression levels of HIF-1 α and its downstream target genes in myocardial tissues.

MATERIALS AND METHODS

Animals

Sixty-day-old male SD rats (n = 48) were purchased from Beijing Huafukang Bio-technology Co., Ltd., weighing 240-260 g. After purchase, they were acclimatised and

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kept for more than one week, with the room temperature maintained at 22-25°C and fed freely. Twelve hours before the experiment, the rats were fasted and freed to drink water. All animal experiments in this research were performed in compliance with the Guide for the Care and Use of Animal Ethics and approved by the Experimental Animal Ethics Committee of the Affiliated Hospital of Chengde Medical University.

Experimental protocol

The rats were anaesthetized intraperitoneally with sodium pentobarbital (50 mg/kg, SIGMA), the hair on the back and abdomen was clipped, and the rats were placed in the rectangular openings of the prefabricated template, exposing the bare skin while protecting the remaining skin. The rats were randomly divided into 3 groups (n=16): (1) the sham group: a 37°C water bath was used for 15 seconds on the back and 8 seconds on the abdomen and 0.25 ml of saline was injected intraperitoneally after immersion. (2) the scald group: in a 100°C water bath, the back was immersed for 15 s, the abdomen was immersed for 8 s after scalding and 0.25 ml saline was injected intraperitoneally; (3) the scald +VPA group: VPA treatment (300 mg/kg dissolved in 0.25 ml 0.9% saline) was given intraperitoneally after scald; each large group was divided into two subgroups (n=8) according to the two observation time points of scald 3h and scald 6h. Blood was collected through the abdominal aorta and isolated left ventricular myocardial tissue was maintained in 4% paraformaldehyde in a -80°C refrigerator (Tang *et al.*, 2018).

Plasma collection and detection

Abdominal aortic puncture was performed at 3h and 6h post-injury, and blood specimens were obtained from 8 rats at each time point; blood specimens were centrifuged to obtain plasma, and plasma levels of creatine kinase isoenzymes (CK-MB) were determined by a fully automated biochemical analyzer (Hitachi, Japan) (Luo *et al.*, 2014).

Cardiomyocyte apoptosis rate

Each rat left ventricular tissue was fixed in 4% paraformaldehyde for 24 hours, paraffin embedded, 5 μ m thickness section, xylene dewaxing, gradient ethanol hydration, proteinase K working solution for 20 min, TUNEL (Nanjing Kaiji Biotechnology Development Co., Ltd.) reaction solution 37°C in a wet box incubated for 60 min away from light, PBS rinsing and then fluorescence microscope observation and photography. Five non-overlapping 400-high magnification fields of view in the myocardial tissue area were randomly selected, and the number of apoptotic cardiomyocytes and the total number of cardiomyocytes were counted and the apoptosis rate of cardiomyocytes=number of apoptotic cardiomyocytes/total number of cardiomyocytes \times 100% (Shen *et al.*, 2021).

ELISA

Weigh 50mg of frozen myocardial tissue, and cell lysate was added to the homogenate and centrifuged at 275g for 15 minutes at 4°C. Protein concentration was measured by the diquinoline carboxylic acid method, caspase-3 activity (Abcam) and NO content were determined by a diquinone assay (Nanjing Kaiji Bioengineering Co., Ltd.) in accordance with the instructions of the kit (Guan *et al.*, 2022; Yalcin *et al.*, 2020).

Western Blot

Weigh 100 mg of frozen myocardial tissue at 6 h post-injury and 1 mL of RIPA lysis solution was added to the homogenate, which was left on ice and then centrifuged at 275 g for 20 min. The supernatant was aspirated and used for the determination of protein concentration by the bicinchoninic acid assay. After routine polyacrylamide gel electrophoresis, membrane transfer, and closure with 5% skimmed milk powder for 60 min, the corresponding primary antibodies were added: mouse anti-rat GAPDH monoclonal antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., 1:5 000), rabbit anti-rat HIF-1 α (Abcam, 1:2 000), rabbit anti-rat iNOS (Abcam, 1:400), rabbit anti-rat BNIP3(Abcam,1:100), rabbit anti-rat caspase-3 (Cell Signalling, 1:1000), incubated at 4°C overnight; TBST washed 15 min \times 3 times and then the corresponding secondary antibody (1:5 000) was added, respectively, incubated at room temperature for 30 min; TBST washed 15 min \times 3 times, and luminescence was performed with ultrasensitive ECL luminescent solution. Exposure, development, and fixation were performed, and the grey level of the bands was analysed by Image J software (National Institutes of Health, USA) (Feng *et al.*, 2021).

STATISTICAL ANALYSIS

SPSS 23.0 statistical software (IBM Corp., Armonk, NY, USA) to process the data. The results of each index were expressed as mean \pm standard deviation. Comparisons between groups were made by one-way ANOVA, and two independent samples were analyzed by t-test. P<0.05 was considered statistically significant.

RESULTS

CK-MB levels

As shown in fig. 1, the levels of CK-MB were significantly higher in the scald group compared to the sham group (both P<0.05), while CK-MB levels were significantly lower in the scald + VPA group than in the scald group (both P<0.05).

TUNEL staining

After scald injury, apoptosis of cardiomyocytes, edoema and widening of the interstitial space, disordered myofibrillar arrangement and fracture were observed, and

the myocardial injury gradually increased with time; whereas the pathological changes in the scald + VPA group were significantly reduced compared with those in the scalded group, with the number of apoptotic cardiomyocytes significantly reduced and myofibrillar arrangement in a neat manner (fig. 2A). The cardiomyocyte apoptosis rate was significantly higher in the scald group than in the sham group (both $P < 0.05$), and the longer the post-scalded time, the higher the rate of cardiomyocyte apoptosis, whereas the apoptosis rate of cardiomyocytes in the scald + VPA group were significantly reduced compared with that in the scald group (both $P < 0.05$) (fig. 2B).

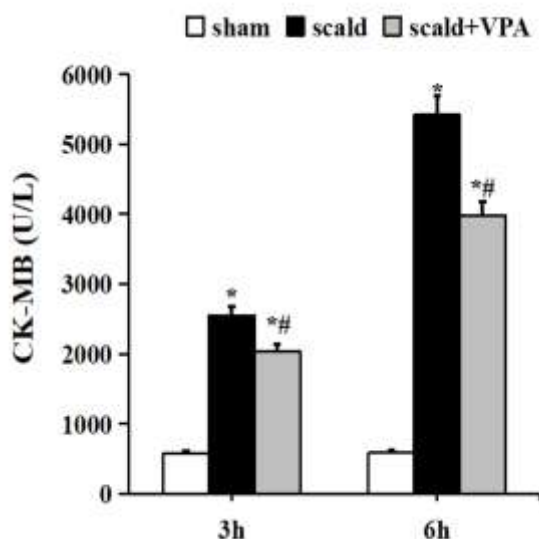


Fig. 1: Effect of VPA on CK-MB levels. CK-MB levels were measured 3 and 6 hours after scald, the values were expressed as the mean (SD), $n=8$. * $P < 0.05$ compared with the sham group; # $P < 0.05$, compared with the scald group.

Caspase-3 activity and NO content

We observed the changes of caspase-3 activity (fig. 3A) and NO content (fig. 3B) in myocardial tissue by ELISA. At 3h and 6h after injury, the caspase-3 activity and NO content of the scald group were significantly higher than that of the sham group; while the caspase-3 activity and NO content of the scald + VPA group were significantly lower than that of the scald group.

Expression of HIF-1 α , BNIP3, iNOS and caspase-3

Fig. 4 shows the HIF-1 α pathway and caspase-3 protein expression in myocardial tissue by Western Blot method. The protein expression of HIF-1 α and its downstream target genes BNIP3, iNOS and caspase-3 were significantly increased in the scald group compared with the sham group (all $P < 0.05$) and the protein expression of HIF-1 α , BNIP3, iNOS and caspase-3 were significantly decreased in the scald + VPA group compared with the scald group (all $P < 0.05$), which were statistically significant.

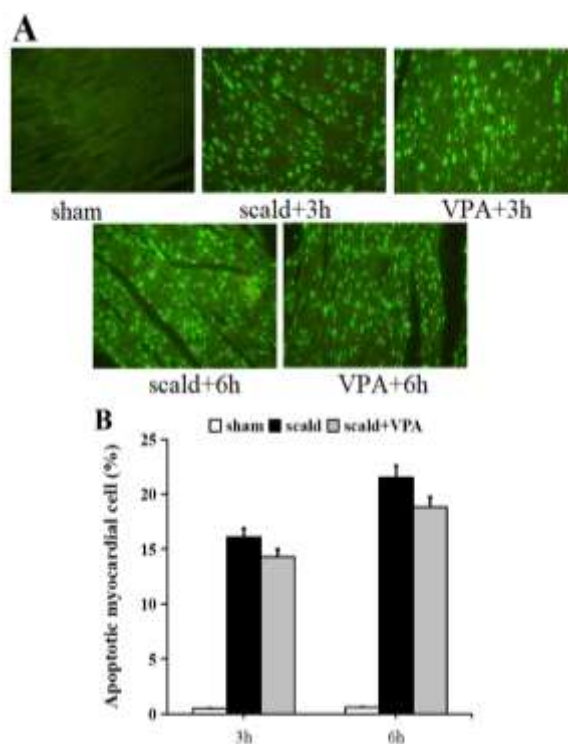


Fig. 2: Effect of VPA on cardiomyocyte apoptosis. Cardiomyocyte apoptosis was observed by Tunel staining method ($\times 400$) at 3 and 6 hours after scald (A), the number of apoptotic cardiomyocytes and the total number of cardiomyocytes were counted and the ratio of cardiomyocyte apoptosis was counted (B) and the values were expressed as the mean (SD), $n=8$. * $P < 0.05$, compared with the sham group; # $P < 0.05$, compared with the scald group.

DISCUSSION

In severe burns, cardiac output decreases by more than 50% after 10 min and less than 1/3 after 60 min. Therefore, myocardial ischemia, hypoxia damage, and decompensation occur before blood volume decreases significantly due to the increase in capillary permeability after severe burns (Huang, 2016). Myocardial apoptosis caused by burn injury is an important pathological mechanism for the occurrence and development of myocardial injury and its complications and has a temporal correlation with the occurrence of cardiac function inhibition (Carlson *et al.*, 2002; Williams *et al.*, 2011; Barrow *et al.*, 2000). Inhibition of cardiomyocyte apoptosis in the early stage of burn injury is conducive to the protection of cardiac function and the improvement of cardiomyocyte tolerance to ischaemia and hypoxia.

Sodium valproate, a short-chain fatty acid-based histone deacetylase inhibitor, increased myocardial histone H3 acetylation, inhibited the expression of cysteine aspartate protease 3 and significantly reduced the activity of CK-MB in the myocardium of lethally ill rats (Luo *et al.*,

2014); VPA protected cardiomyocytes of rats in hemorrhagic shock from hemorrhagic and hypoxic stress via the Akt/BCL-2 survival pathway (Wang *et al.*, 2016); VPA significantly reduced cardiac injury after myocardial infarction in rats by affecting the Foxm1 signalling pathway (Tian *et al.*, 2019). In the present experiment, it was also demonstrated that VPA significantly reduced CK-MB activity and attenuated myocardial injury after lethal scald injury in rats. Pathological results further showed that VPA reduced interstitial oedema, myocardial fibre disarrangement and myocardial tissue rupture.

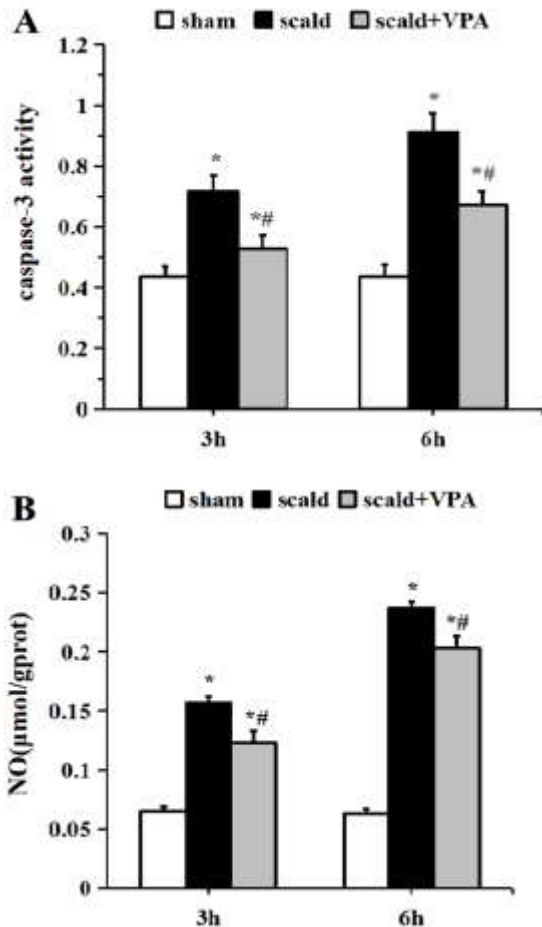


Fig. 3: Effect of VPA on caspase-3 activity and NO content in myocardial tissue. Caspase-3 activity(A) and NO content(B) were measured at 3 and 6 hours after scald, the values were expressed as the mean (SD), n = 8. * $P < 0.05$, compared with the sham group; # $P < 0.05$, compared with the scald group.

Previous studies have shown that in ischaemia/hypoxia-induced cardiomyocyte injury, HIF-1 α acts as a sensitive protein in the hypoxic response and the increased expression of HIF-1 α protein after injury causes damage to cardiac function and apoptosis of cardiomyocytes (Wang X *et al.*, 2012). In this study, we showed that HIF-1 α expression was significantly increased in myocardial tissues after lethal scald injury and after administration of

VPA, HIF-1 α expression was significantly decreased compared with the scald group, indicating that VPA inhibited HIF-1 α expression, thereby inhibiting cardiomyocyte apoptosis. iNOS and BNIP3, as the downstream target genes of HIF-1 α , have been confirmed by previous studies that tissues or cells, under the conditions of ischemia and hypoxia, the HIF-1 α expression increases, accompanied by increased expression of its downstream target genes iNOS, BNIP3 (Jung *et al.*, 2000; He *et al.*, 2023).

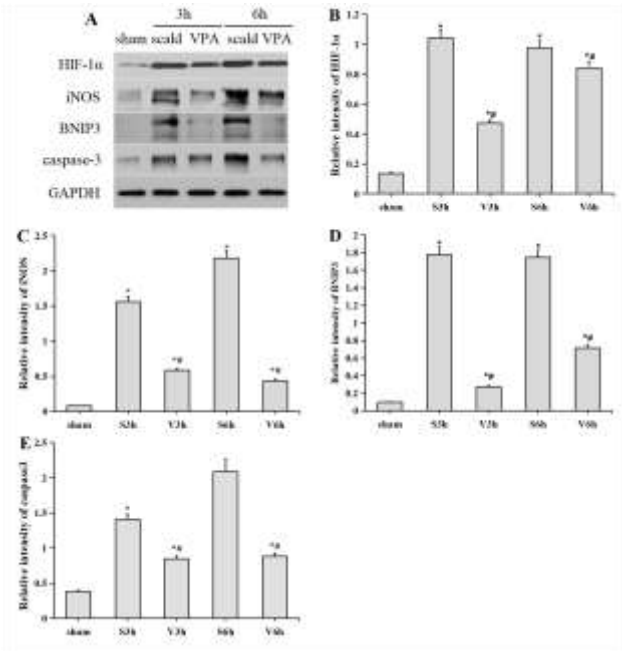


Fig. 4: Effect of VPA on the expression of HIF-1 α , BNIP3, iNOS and caspase-3 in myocardial tissue. Representative immunoblots (A) and semiquantification of HIF-1 α (B), iNOS (C), BNIP3 (D) and caspase-3 (E) in myocardial tissues at 3 and 6 h after injury are shown. Each protein band was normalized to its respective glyceraldehyde 3-phosphate dehydrogenase (GAPDH) band, the values were expressed as the mean (SD), n=3. * $P < 0.05$, compared with the sham group; # $P < 0.05$, compared with the scald group.

During ischemic-hypoxic myocardial injury, iNOS expression increases and acts as a catalytic enzyme for NO synthesis, generating a large amount of NO (Kingery *et al.*, 2017; Jeddi *et al.*, 2017) and high levels of NO in myocardial tissues induce apoptosis in cardiomyocytes through activation of various apoptotic pathways (Zhao *et al.*, 2015). HIF-1 mainly consists of an unstable HIF-1 α and HIF-1 β , and HIF-1 α is transferred to the nucleus and binds to HIF-1 β to activate BNIP3 and regulate certain functions (Semenza, 2001); BNIP3 belongs to the Bcl-2 superfamily of proteins, and is a mitochondrial pro-apoptotic protein, which exerts its pro-apoptotic function after heterodimerization with anti-apoptotic proteins through the BH3 structure (Madhu *et al.*, 2020). In this

experiment, the levels of HIF-1 α , iNOS, BNIP3 and NO were significantly higher in the scald group after injury compared with the sham group and increases with time post-injury; whereas were significantly lower after VPA treatment was given, indicating that VPA inhibited the expression levels of HIF-1 α and downstream target genes in the myocardium of lethally scalded rats. Caspase-3 is a marker of early apoptosis to reflect apoptosis of cardiomyocytes after injury and VPA similarly reduced the activity and protein expression of caspase-3; in the meantime, the rate of apoptosis of cardiomyocytes correlated with caspase-3 positively.

CONCLUSION

In summary, this study demonstrated that VPA protected cardiomyocytes from hypoxic injury by inhibiting cardiomyocyte apoptosis and attenuating myocardial injury via the HIF- α pathway. These results suggested a novel mechanism of VPA-mediated protection of cardiomyocytes in the early stage of lethal scald injury and suggested the potential use of HDACIs in the treatment of lethal scald myocardial injury.

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