Sleep and Breathing (2019) 23:711–717
https://doi.org/10.1007/s11325-018-1739-y

HYPOXIA • ORIGINAL ARTICLE

4-phenylbutyric acid attenuates endoplasmic reticulum stress-mediated apoptosis and protects the hepatocytes from intermittent hypoxia-induced injury

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Received: 21 July 2018 / Revised: 27 September 2018 / Accepted: 8 October 2018 / Published online: 15 October 2018

Abstract

Purpose To investigate the effect of 4-phenylbutyric acid (4-PBA) on intermittent hypoxia (IH)-induced liver cell injury and to clarify the underlying mechanisms.

Methods L02 cells (normal human liver cells) were cultured in normoxic condition or subjected to intermittent hypoxia for 4, 8, and 12 h. A part of hypoxia-treated L02 cells was applied with 4-PBA 1 h before exposure to hypoxia. The effect of 4-PBA on liver injury, hepatocyte apoptosis, endoplasmic reticulum stress (ERS), and PERK-eIFa2-ATF4-CHOP apoptotic pathway was investigated.

Results (1) IH caused apoptosis in hepatocyte; (2) IH caused ERS in hepatocyte; (3) IH caused hepatic injury; (4) 4-PBA attenuated IH-induced liver cell injury; (5) 4-PBA protected liver cell from IH-induced apoptosis; (6) 4-PBA suppressed ERS-related apoptotic pathway (PERK-eIFa2-ATF4-CHOP), but did not suppress IH-induced unfold protein reaction (UPR).

Conclusions 4-PBA could protect liver cells by suppressing IH-induced apoptosis mediated by ERS, but not by reducing the UPR.

Keywords Intermittent hypoxia · Liver injury · Endoplasmic reticulum stress · Apoptosis · 4-phenylbutyric acid

Introduction

Obstructive sleep apnea (OSA) syndrome is a kind of sleep breathing disorder which is characterized by repeated partial or complete upper airway collapse leading to recurrent and intermittent hypoxia during sleep [1], eventually leading to chronic intermittent hypoxia (IH) [2]. Recent studies have shown that the characteristic chronic IH of OSA may be an independent risk factor for liver injury [3–7].

Accumulation studies have proved that chronic IH could induce endoplasmic reticulum stress (ERS) [8–10]. As a way of responding to the homeostasis of the internal environment in eukaryotic cells, ERS has attracted more and more attention. Many factors can induce the occurrence of ERS, such as hypoxia, ischemia-reperfusion, alcohol, drugs, poisoning, infection (bacteria, virus, etc.), ultraviolet radiation, and lack of nutrients [11]. ERS can cause unfolded protein reaction (UPR), and UPR maintains the homeostasis of endoplasmic reticulum by reducing the synthesis of new proteins, increasing the synthesis of chaperones and the degradation of misfolded or unfolded proteins [12]. Studies have shown that hypoxia can induce ERS, and UPR may be a way for cells to cope with hypoxic stimulation [13–15].

Liver cells are rich in endoplasmic reticulum, which can synthesize and secrete many kinds of proteins. It has been reported that ERS/UPR may play an important role in a variety of liver diseases [16], including alcohol-induced liver injury, nonalcoholic fatty liver disease, hepatic insulin resistance, ischemia-reperfusion injury, and other acute hepatotoxins [17–21]. For cells, the UPR caused by ERS under various stresses is a protective measure, but when the UPR cannot maintain the homeostasis of endoplasmic reticulum, persistent and excessive ERS can cause apoptosis [22–24]. 4-phenylbutyric acid (4-PBA) is a molecular chaperone which can stabilize protein conformation and improve protein-folding function of endoplasmic reticulum. Recently, it has

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been found that 4-PBA can alleviate the apoptosis mediated by ERS [25, 26].

Therefore, we hypothesized that 4-PBA could reduce IH-induced liver injury by attenuating ERS-mediated apoptosis. In the present study, L02 cells were pre-treated with 4-PBA and exposed to IH; the activity of liver enzymes, hepatocyte apoptosis, and ERS of liver cells was analyzed to evaluate the effect of 4-PBA on IH-induced liver injury.

Materials and methods

Materials

RPMI 1640 and fetal bovine serum (FBS) were obtained from Thermo-Fisher Scientific (Shanghai, China). 4-PBA was obtained from Solarbio Life Science (Beijing, China). Antibodies against caspase-12, glucose-regulated protein 78 (GRP78), and GADPH were purchased from Abcam (Shanghai, China). Antibodies against caspase-3, phosphorylated-PKR-like ER protein kinase (p-PERK), phosphorylated-eukaryotic translation initiation factor 2 subunit α (p-eIF2α), activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) were purchased from Cell Signaling Technology (Shanghai, China).

Cells

The human hepatic cell line L02 was obtained from China Center for Type Culture Collection (Wuhan, China). L02 cells were cultured in medium mixed with RPMI 1640, 10% (*v*/*v*) FBS, and 100 units/ml penicillin (in 5% CO_2, 37 °C) and passaged every 3–5 days. Then cells were grown under normoxic condition (control group, 21% O_2) for 12 h or intermittent hypoxic (IH group, 1.5% O_2 10 min then 21% O_2 5 min) condition for 4, 8, and 12 h at 37 °C/5% CO_2. IH exposure was conducted using a custom-designed computer-controlled incubator chamber. A part of IH group L02 cells was applied with 4-PBA (10 mM) 1 h before exposure to hypoxia to reduce the effect of ERS. The 4-PBA was added into the culture medium.

Biochemical assays

Culture medium alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentration (U/L) were determined by commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the user's instructions.

Protein extraction

After hypoxic or normoxic incubation, the cells were washed with ice-cold phosphate-buffered saline (PBS) for two times then lysed in 1 ml of lysis buffer [33 mM Tris, 165 mM NaCl, pH 7.5, 1% Triton X-100, 10% glycerol, 50 μl of 100 mM phenylmethanesulphonylfluoride (PMSF), and 50 μl of protease inhibitor] on ice. Cell lysates were boiled for 5 min and centrifuged at 12000*g* for 10 min at 4 °C, and the supernatants were retained; the supernatants were subjected to western blotting for caspase-3, caspase-12, GRP78, ATF4, CHOP, phospho-PERK, and phospho-eIF2α.

Western blot analysis

For western blot, 4–20% polyacrylamide gradient gel (SDS-PAGE) was used to analyze the supernatant containing 20 μg protein. The separated proteins were subsequently transferred to nitrocellulose membranes. These membranes were blotted with the corresponding antibodies after blocking with Tris saline buffer containing 5% dry milk and 0.1% Tween 20 for 1 h. The primary antibodies were as follows: rabbit anti-human GRP78, rabbit anti-human caspase-3, caspase-12, rabbit anti-human ATF4, CHOP, phospho-PERK and phospho-eIF2α, and rabbit anti-human GADPH. The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase. A chemiluminescence detection system was used to detect the membranes. The band intensity was measured densitometrically and was normalized to the level of GADPH.

Statistical analysis

Results are expressed as mean ± SD for *n* independent observations as indicated. Statistical analyses were performed using Student's *t* test by SPSS 24.0. *P* value less than 0.05 was considered statistically significant difference.

Results

IH caused apoptosis in hepatocyte

The levels of caspase-3 and cleaved caspase-12 were determined to evaluate the effect of IH on hepatic apoptosis. As shown in Fig. 1a and b, the levels of caspase-3 and cleaved caspase-12 significantly elevated after 4 h, 8 h, and 12 h IH ($P < 0.05$), and similarly, the caspase-3 and cleaved caspase-12 level of IH 12-h group was significantly increased compared with other three groups ($P < 0.05$).

IH caused ERS in hepatocyte

The level of molecular chaperone GRP78 was detected to evaluate the role of IH on hepatic ERS. GRP78 was increased after 4 h, 8 h, and 12 h IH compared with NC group ($P < 0.05$), and GRP78 level of IH 12-h group was significantly increased

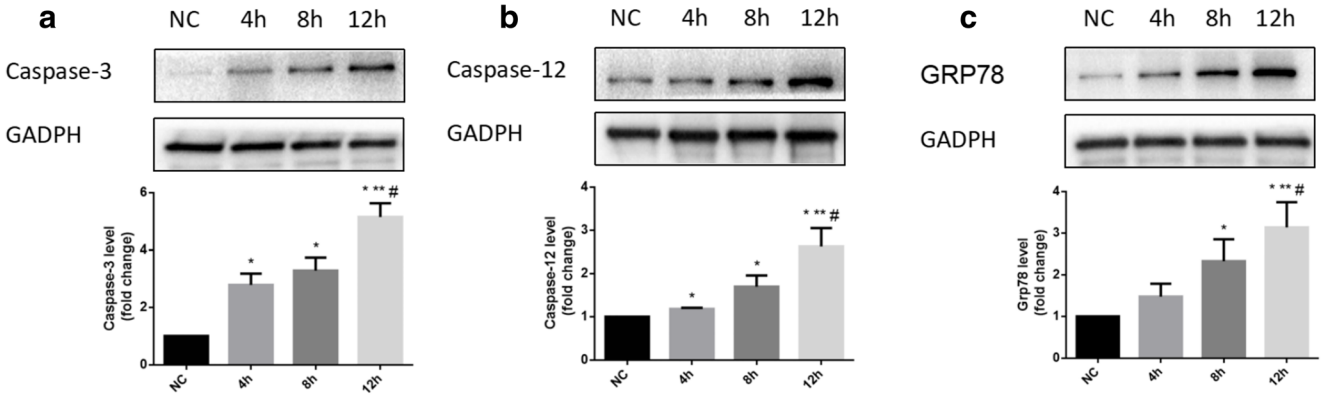

Fig. 1 Hepatic apoptosis and ERS after IH (4 h, 8 h, and 12 h). The protein levels of **a** cleaved caspase-3, **b** cleaved caspase-12, and **c** GRP78 were detected by western blotting. *$P < 0.05$ vs. NC group, **$P < 0.05$ vs. IH 4-h group, #$P < 0.05$ vs. IH 8-h group. NC, negative control; GRP78, glucose-regulated protein 78

compared with other three groups ($P < 0.05$) (Fig. 1c). This result indicated that IH caused hepatic ERS.

IH caused hepatic injury

Culture medium ALT and AST were determined to evaluate the effect of IH on hepatic injury. As shown in Fig. 2, the levels of ALT and AST were significantly elevated after 12 h IH ($P < 0.05$). These results showed that IH caused apoptosis and hepatic injury.

4-PBA attenuated liver cell injury induced by IH

As shown in Fig. 2, the levels of ALT and AST in the IH group were significantly elevated when compare to that in the NC group ($P < 0.05$), but these were significantly decreased in IH + 4-PBA group when compared to the IH group ($P < 0.05$). These findings demonstrated that 4-PBA attenuated liver injury induced by IH.

4-PBA protected liver cell from IH-induced apoptosis

After 12-h exposure to IH, the expression of two important biochemical markers of apoptosis, caspase-3 and cleaved caspase-12, was evaluated using western blot (Fig. 3a, b). Exposure to IH significantly upregulated the protein levels of these two makers ($P < 0.05$), while these changes were evidently restored by the treatment of 4-PBA ($P < 0.05$). These results suggested that 4-PBA protected the liver from IH-induced liver cell apoptosis.

4-PBA suppressed ERS-related apoptotic pathway

To delineate the role of 4-PBA on IH-induced ERS/UPR, western blot was used to evaluate the protein levels of GRP78. As shown in Fig. 4a, IH increased the protein level of GRP78 ($P < 0.05$); however, this protein was not significantly reduced in the IH + 4-PBA group when compared to that in the IH group ($P > 0.05$). With the unchanged level of GRP78, we further investigated the signal molecules of ERS-associated apoptotic pathways (Fig. 4b–e). There was an evident increase of the p-PERK, p-eIF2α, ATF4, and CHOP in IH group when compared to the NC group ($P < 0.05$). However, these changes of protein levels were all significantly reversed by the treatment of 4-PBA ($P < 0.05$). Therefore, the present findings demonstrated that 4-PBA did not suppress IH-induced UPR but suppressed ERS-associated apoptosis of the liver cell by inhibiting the PERK-eIF2α-ATF4-CHOP pathway.

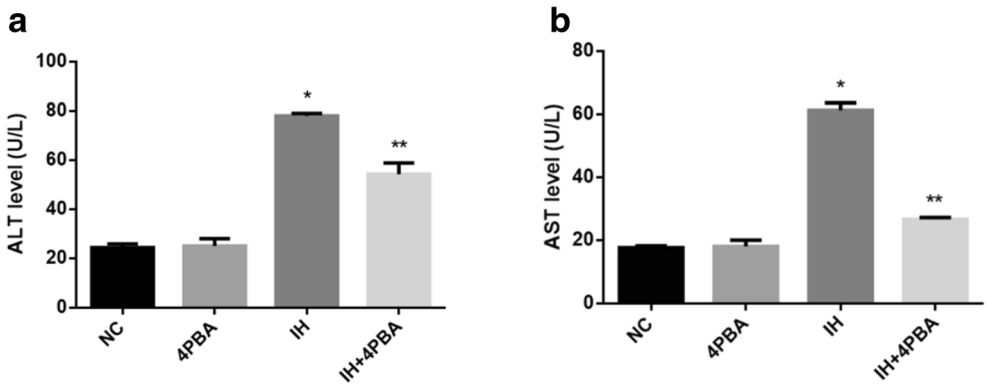

Fig. 2 Culture medium biochemical indicator levels. The biochemical indicators of liver function **a** ALT and **b** AST were detected by commercial kits. *$P < 0.05$ vs. NC group, **$P < 0.05$ vs. IH group. NC, negative control; IH, intermittent hypoxia; 4PBA, 4-phenylbutyric acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase

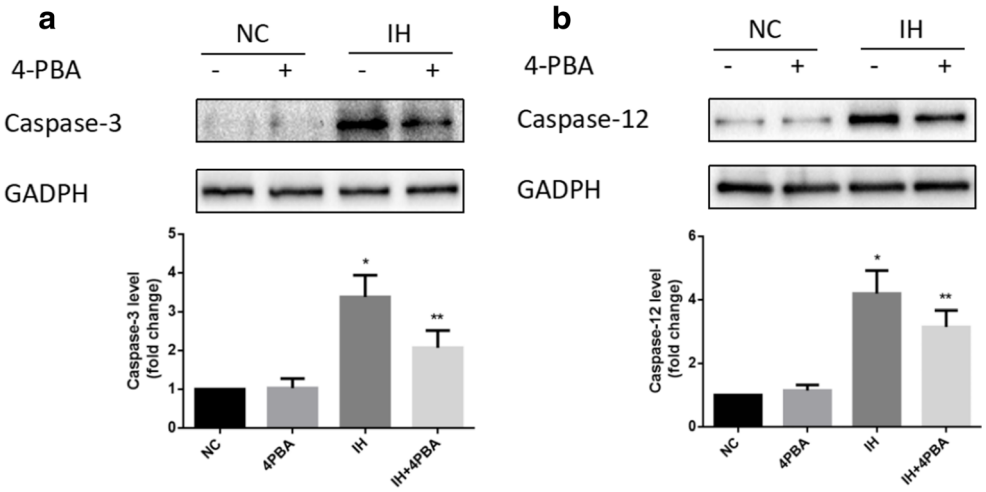

Fig. 3 Apoptosis of hepatocytes. The protein levels of **a** caspase-3 and **b** cleaved caspase-12 were detected by western blotting. $*P < 0.05$ vs. NC group, $**P < 0.05$ vs. IH group. NC, negative control; IH, intermittent hypoxia; 4PBA, 4-phenylbutyric acid

Discussion

In the present study, L02 cells were exposed to IH for 4, 8, and 12 h and treatment with 4-PBA. It was observed that GRP78, caspase-3, and caspase-12 elevated at most after 12-h IH with statistical significance compared to NC, 4-h and 8-h groups; therefore, we chose 12-h IH exposure to evaluated the effect of 4-PBA on IH-induced liver injury. The concentration of 4-PBA is determined by previous study [27].

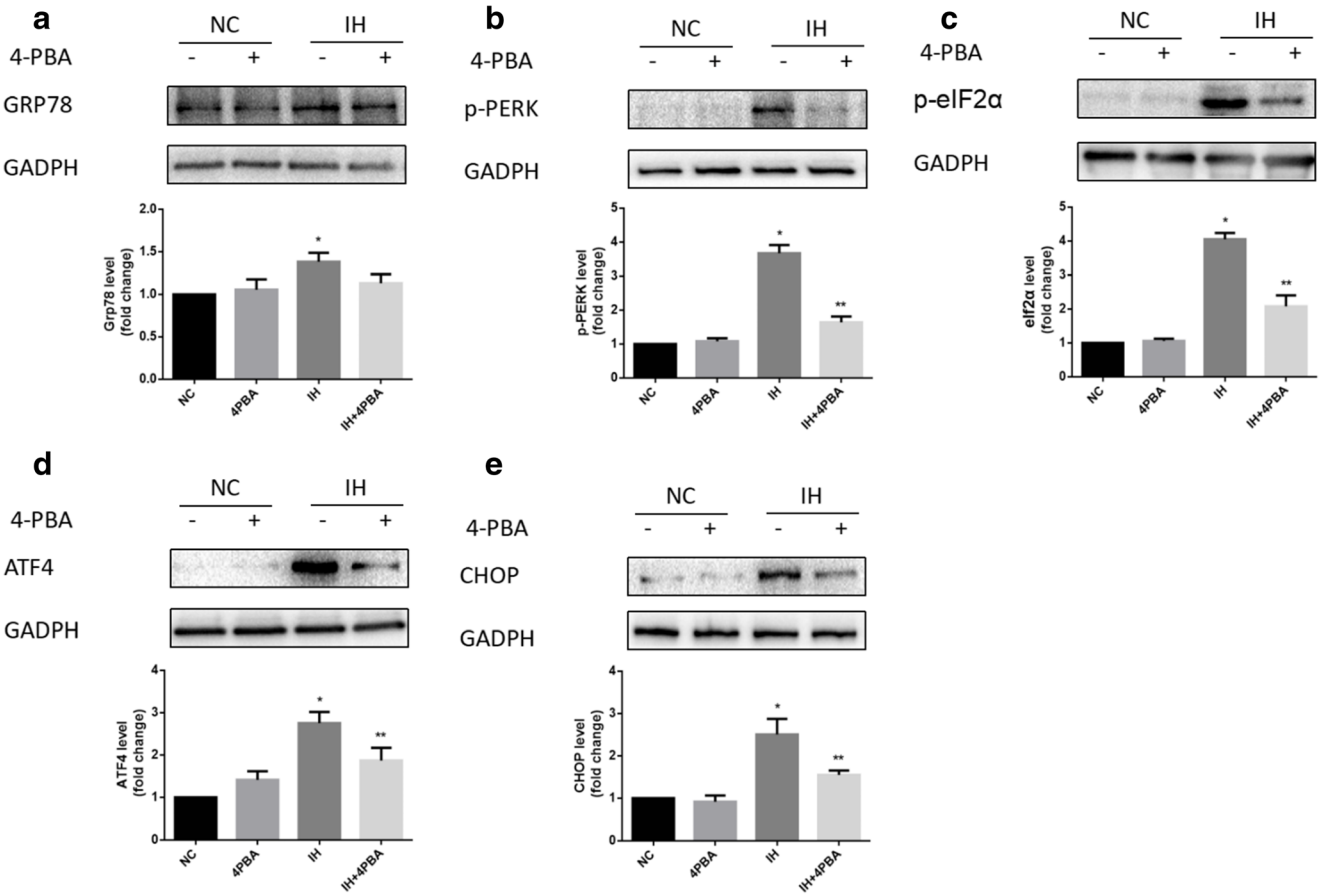

Fig. 4 ERS-related apoptotic pathway. The protein levels of **a** GRP78, **b** p-PERK, **c** p-eIF2α, **d** ATF4, and **e** CHOP were detected by western blotting. $*P < 0.05$ vs. NC group, $**P < 0.05$ vs. IH group. NC, negative control; IH, intermittent hypoxia; 4PBA, 4-phenylbutyric acid; GRP78, glucose-regulated protein 78; p-PERK, phosphorylated-PKR-like ER protein kinase; p-eIF2α, phosphorylated-eukaryotic translation initiation factor 2 subunit α; ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein

It is widely recognized that OSA and its associated chronic IH is related to the pathogenesis of various liver diseases [28–30]. The result of animal experiment showed that the serum ALT level of mice exposed to IH for 12 weeks was two times that of the control group, and the swelling and deformation of hepatocytes appeared [31]. Clinical studies found that ALT and AST in OSA patients were higher than those in control group [32–34], and the severity of liver injury was related to the severity of OSA [35, 36]. A meta-analysis showed that 13.3% of OSAHS patients were associated with elevated ALT and 4.4% with elevated AST [37]. So there is a view that OSA is an independent risk factor for liver injury [38, 39]. 4-PBA can recover liver function from injuries induced by drugs and lipid accumulation [40–42]; however, whether 4-PBA has protective effects against IH-induced liver injury remains unknown. The present results showed that 4-PBA significantly protected liver from IH-induced injury, as evidenced by reduced culture medium ALT and AST levels.

Animal models and clinical data suggested that apoptosis is an important mechanism of IH-induced tissue or organ injuries [43–45]. Apoptosis of hepatocyte could be observed in the majority of types of human liver diseases, including hepatic ischemia-reperfusion injury, fibrosis, nonalcoholic liver diseases, alcoholic liver disease, and hepatocellular carcinoma [46]. It was reported that exposure to IH significantly inhibited the proliferation and accelerated apoptosis of human liver cells [47]. In the present study, the protein level of caspase-3 and cleaved caspase-12 was obviously increased after 12-h IH. The expression of caspase-3 and cleaved caspase-12 is related to apoptosis [48, 49]. Caspase-12 was a specific enzyme in the process of ERS-mediated apoptosis and was activated to cleaved caspase-12 during this process [50]. In contrast, the upregulation of these two apoptosis markers was significantly attenuated by 4-PBA treatment. These results are consistent with previous data showing that 4-PBA could protect hepatocytes from chemical agents-induced apoptosis [40, 41, 51].

ERS may be activated by various disturbances, including IH [10, 13, 52]. GRP78 is a molecular chaperone of the endoplasmic reticulum and is a sensitive marker of UPR response during ERS [53]. In the state of ERS, GRP78 was released and the transmembrane proteins, PERK, ATF6, and IRE-1, were activated to trigger three different UPR branches to protect endoplasmic reticulum from severe damage [54]. When ERS was excessive and prolonged, the downstream apoptotic proteins were activated and turned to be executioners [55]. PERK-eIF2α-ATF4-CHOP was an important pathway by which excessive ERS lead to cell apoptosis [56, 57]. The accumulation and over expression of CHOP could upregulate the apoptotic gene Bax and downregulate the anti-apoptotic gene Bcl-2; this would lead to the release of cytochrome C from the mitochondrial membrane space to the cytoplasm and finally induce cell apoptosis [58, 59]. In the present study, the PERK-eIF2α-ATF4-CHOP apoptotic pathway was activated by IH and was significantly reduced by 4-PBA treatment, but the IH-induced increase of GRP78 was not significantly reduced by 4-PBA treatment. These results suggested that treatment of 4-PBA could protect liver cells by suppressing IH-induced apoptosis mediated by ERS, but not by reducing the UPR. Although the previous results of 4-PBA to reduce the hepatocyte apoptosis are consistent, the results of 4-PBA on GRP78 are not consistent. Some study showed that 4-PBA could reduce GRP78 and inhibit ERS [27], but another study failed to produce similar results [60]. This needs to be further studied.

Conclusion

Altogether, the present data demonstrated that 4-PBA protected liver cell from IH-induced hepatocyte apoptosis by suppressing the activation of PERK-eIF2α-ATF4-CHOP apoptotic pathway initiated by ERS. This finding indicated a liver-protective effect of 4-PBA in IH-induced liver injury, and it is hypothesized that this effect is mediated at least partly by inhibiting the ERS-induced apoptosis.

Funding National Natural Science Foundation Committee of China provided financial support in the form of National Natural Science Foundation of China funding (No. 81670086, No. 81370183). Natural Science Foundation Committee of Tianjin City provided financial support in the form of Natural Science Foundation of Tianjin City funding (No. 14JCYBJC27800) and major special project for the prevention and control of chronic diseases in Tianjin (Grant No. 17ZXMFSY00080). The sponsor had no role in the design or conduct of this research.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

For this type of study formal consent is not required.

Abbreviations 4-PBA, 4-phenylbutyric acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATF4, activating transcription Factor 4; CHOP, C/EBP homologous protein; ERS, endoplasmic reticulum stress; FBS, fetal bovine serum; GRP78, glucose-regulated protein 78; IH, intermittent hypoxia; NC, negative control; OSA, obstructive sleep apnea; PBS, phosphate-buffered saline; p-eIF2α, phosphorylated-eukaryotic translation initiation factor 2 subunit α; PMSF, phenylmethanesulphonylfluoride; p-PERK, phosphorylated-PKR-like ER protein kinase; UPR, unfold protein reaction

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Comment

This study shows that increasing exposure of hepatocytes to hypoxia demonstrates hypoxia causes a dose-dependent injury which is partly attenuated by 4-phenylbutyric acid. The role of intermittent hypoxia in the pathophysiology of OSA-related liver dysfunction is clearly of interest to scientists and clinicians alike; this study helps advance our understanding of this area and potential therapeutic pathways.

While this is an in vitro study, it does provide support for the notion that currently accepted adequate notions of treatment efficacy may need to be reconsidered. Perhaps with CPAP, some is helpful, more is better, and most of the night is better still.

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