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Original Research Article

Hypaphorine ameliorates lipid accumulation and inflammation in a cellular model of non-alcoholic fatty liver by regulating p38/JNK and NF-κB signaling pathways

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Abstract

Purpose: *To investigate the therapeutic effect and underlying mechanism of hypaphorine in a cellular model of non-alcoholic fatty liver disease (NAFLD).*
Methods: *Palmitic acid (PA) was used to induce a NAFLD phenotype in hepatocytes. Cell viability and apoptosis were evaluated by CCK-8 and flow cytometry assays. Inflammatory response was measured by enzyme-linked immunosorbent assay (ELISA). The effect of hypaphorine on lipid accumulation was evaluated using Oil Red O staining and triglyceride kits. Activation of p38/c–Jun N-terminal kinase (JNK) and NF-κB pathways were analyzed by immunoblot assay.*
Results: *Hypaphorine significantly improved cell viability ($p < 0.01$), suppressed inflammatory response ($p < 0.01$), and reduced lipid accumulation ($p < 0.01$) in PA-treated hepatocytes. Hypaphorine ameliorated lipid accumulation and inflammation in PA-treated hepatocytes by targeting p38/JNK and NF-κB pathways.*
Conclusion: *Hypaphorine may serve as a therapeutic target in NAFLD. However, in vivo studies to validate this finding are required.*

Keywords: *Non-alcoholic fatty liver disease (NAFLD), Inflammation, Lipid accumulation, p38/JNK pathway, NF-κB pathway*

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become the most common liver disease worldwide [1]. Liver fat is physiologically balanced by the influx of non-esterified free fatty acids from plasma, dietary fat intake, fat regeneration, and mitochondrial β -oxidation [2,3]. Imbalanced fat metabolism leads to fat accumulation in the liver, which may be secondary to a fat increase on a high-fat diet [4]. Several factors, including insulin resistance, oxidative stress, impaired autophagy, lipid toxicity, and enterogenic lipopolysaccharides, contribute to NAFLD, resulting in a range of histological abnormalities from simple steatosis to non-alcoholic steatohepatitis (NASH) [5]. There are currently no approved drug treatments for NAFLD.

Hypaphorine is an indole alkaloid isolated from plants of the genus *Erythrina* [6]. Several studies have shown that hypaphorine has potent effects on inflammatory and immune responses. For example, hypaphorine was reported to play an important role in oxidative stress response and sleep induction in mice [7]. Another recent study showed that hypaphorine alleviates inflammatory damage in endothelial cells [8]. In mice, hypaphorine reduced RANKL-induced osteoclast formation and prevented inflammatory bone loss by inhibiting the ERK, P38, JNK, and NF-κB pathways [9]. In acute sepsis-induced lung injury, hypaphorine played an anti-inflammatory role by regulating the DUSP1/P38/JNK pathway. In addition, hypaphorine inhibited the differentiation of 3T3-L1 cells and induced insulin resistance with tamasone [10]. Despite these findings, the possible mechanisms and effects of hypaphorine in palmitic acid (PA)-induced NAFLD have not been reported.

This study was performed to investigate the therapeutic potential and underlying mechanism of hypaphorine in hepatocytes that were treated with Palmitic acid (PA) to simulate NAFLD.

EXPERIMENTAL

Cell culture and treatments

The AML-12 hepatocyte cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM/F12 medium containing 10 % fetal bovine serum (FBS). To construct a model of NAFLD, the cells were stimulated for 24 h with 200 μM PA dissolved in bovine serum albumin and then treated for 24 h with 5, 25, or 50 μM hypaphorine.

Cell Counting Kit-8 (CCK-8) assay

Cell viability was analyzed by CCK-8 assay. Cells on a multi-well plate were treated with 5, 25, or 50 μM hypaphorine for 24 h, mixed with CCK-8 solution (10 μL/well), and cultured for 1 h at 37 °C. The absorbance of each well was then read at 450 nm with a microplate reader.

Cell apoptosis

The percentage of apoptotic cells in each treatment group was determined using an Annexin V/PI Apoptosis Detection Kit in accordance with the manufacturer's protocol (Sigma-Aldrich, USA). The cells were digested and resuspended in a reaction buffer containing Annexin V and propidium iodide for 5 min at room temperature.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6; Beyotime, Beijing, China) in cell lysates were measured with an ELISA kit. All experiments were carried out according to the manufacturer's guidelines.

Oil red O staining

Cells in each well of a multi-well plate were fixed with 10 % formalin and stained with Oil red O (Sigma-Aldrich) to measure intracellular lipid accumulation.

Cellular triglyceride (TG) analysis

The TG content of cells on 6-well plates after treatment for 24 h with PA and hypaphorine was measured with a cellular TG content detection kit (BC0625, Beyotime, Beijing, China) according to the manufacturer's instructions.

Western blot

Total proteins were extracted with RIPA buffer (Beyotime, Shanghai, China). The samples were subjected to 10 % SDS-PAGE, transferred onto PVDF membranes, and blocked with 5 % fat-free milk. Subsequently, the membranes were incubated for 2 h with primary antibodies targeting PPAR-a (1:1000), SREBP1c (1:1000), AMPK (1:1000), p-AMPK (1:1000), p-p65 (1:1000), p65 (1:1000), p-p38 (1:1000), p38 (1:1000), p-JNK (1:1000), JNK (1:1000), and GAPDH (1:10000) all from Abcam. Finally, the membranes were conjugated with secondary antibodies (Abcam) for 1 h. Relative protein expression was measured using ImageJ 9.0 software (USA).

Statistical analysis

GraphPad 6.0 was used for statistical analysis. Three replicates were performed for each experiment. One-way ANOVA and Student's *t*-test were used for statistical comparisons. The threshold for significance in all statistical tests was $P < 0.05$.

RESULTS

Hypaphorine improved the viability of PA-treated hepatocytes

The structure of hypaphorine is shown in Figure 1 A. To evaluate the effect of hypaphorine on hepatocyte viability, AML-12 cells were stimulated with various doses of hypaphorine

and subjected to CCK-8 assay. In normal hepatocytes (i.e., hepatocytes that were not treated with PA to simulate NAFLD), hypaphorine had no significant impact on cell viability, except for a small effect at 100 μM, the highest concentration tested (Figure 1 B). Treatment with PA reduced hepatocyte viability and subsequent treatment with hypaphorine reversed the PA-induced loss of viability in a dose-dependent manner (Figure 1 C). In addition, PA increased the percentage of apoptotic cells, and subsequent hypaphorine treatment reduced the pro-apoptotic effect of PA in a dose-dependent manner (Figure 1 D). Collectively, these results indicate that hypaphorine promotes the viability of PA-treated hepatocytes.

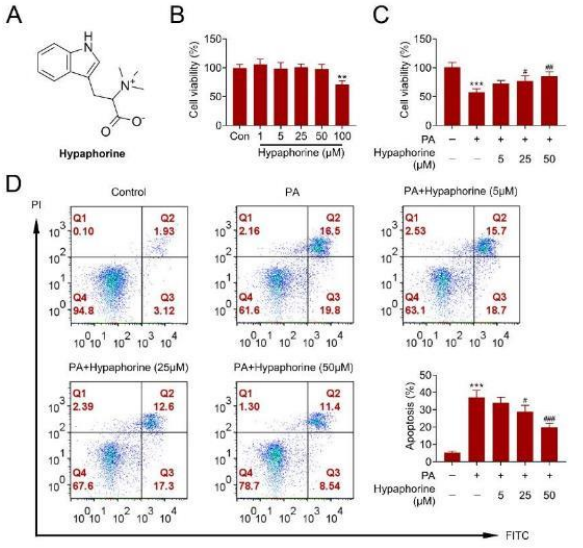

Figure 1: Hypaphorine improves the viability of hepatocytes treated with PA. A: The structure of hypaphorine. B: Viability of AML-12 cells exposed to hypaphorine. C: Viability of AML-12 cells exposed to PA and hypaphorine. D: Apoptosis of untreated control cells and cells exposed to PA alone or PA and hypaphorine was detected by flow cytometry. ***p < 0.001 vs. untreated control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs. PA alone

Hypaphorine improved PA-induced hepatocyte inflammation

Activation of the NF-kB pathway was detected to assess the inflammatory response in PA-treated hepatocytes. Treatment with PA activated p65 phosphorylation, which was reduced by subsequent treatment with hypaphorine (Figure 2 A). Furthermore, PA treatment increased the levels of IL-6, IL-1β, and TNF-α, and subsequent treatment with hypaphorine reduced the levels of these inflammatory cytokines in a dose-dependent manner (Figure 2 B).

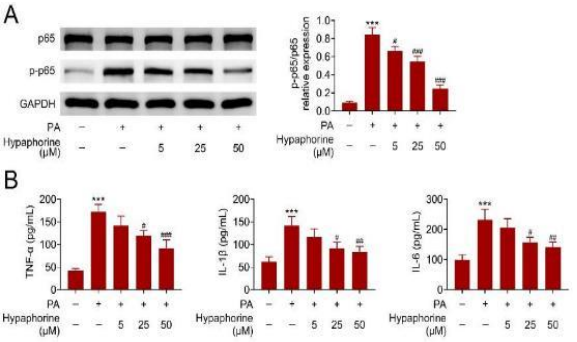

Figure 2: Hypaphorine improved PA-induced cell inflammation. A: PA increased the level of phosphorylated p65 (p-p65), which was reduced by subsequent treatment with hypaphorine. B: The levels of TNF-a, IL-1b, and IL-6 in untreated control cells, PA-treated cells, and cells treated with PA and hypaphorine were analyzed by ELISA. ***P < 0.001 vs. untreated control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs. PA alone

Hypaphorine relieved lipid accumulation in PA-treated hepatocytes

To investigate the effect of hypaphorine on lipid accumulation in hepatocytes, Oil red O staining, and TG detection were performed on AML-12 cells treated with PA and hypaphorine. Oil red O-positivity and TG levels were increased by PA treatment and reduced by subsequent treatment with hypaphorine (Figure 3 A and B). Furthermore, PA treatment increased the level of SREBP1c and reduced the levels of PPAR-a and p-AMPK in AML-12 cells, and subsequent treatment with hypaphorine reversed these PA-induced effects in a dose-dependent manner (Figure 3 C and D). These results suggest that hypaphorine reduces lipid accumulation in hepatocytes.

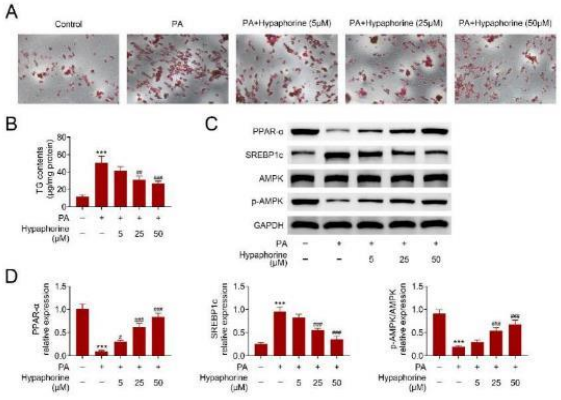

Figure 3: Hypaphorine relieved PA-induced lipid accumulation in hepatocytes. A: Lipid accumulation in untreated control AML-12 cells and AML-12 cells treated with PA alone or PA and hypaphorine was detected by Oil red O staining. B: TG levels in untreated control cells and cells treated with PA alone or PA and hypaphorine were detected by immunoblot

assay. C, D: Levels of SREBP1c, PPAR-a, and p-AMPK in untreated control cells and cells treated with PA alone or PA and hypaphorine were analyzed. ***P < 0.001 vs. untreated control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs. PA alone

Hypaphorine ameliorates lipid accumulation and inflammation

Hypaphorine mediated lipid metabolism and inflammation in PA-treated AML-12 hepatocytes. Therefore, the effect of hypaphorine on the p38/JNK and NF-kB pathways was evaluated. Treatment with PA increased the levels of phosphorylated p38 (p-p38) and JNK (p-JNK), and subsequent treatment with hypaphorine reversed these PA-induced effects in a dose-dependent manner (Figure 4). These results suggest that hypaphorine ameliorated lipid accumulation and inflammation by targeting the p38/JNK and NF-κB pathways.

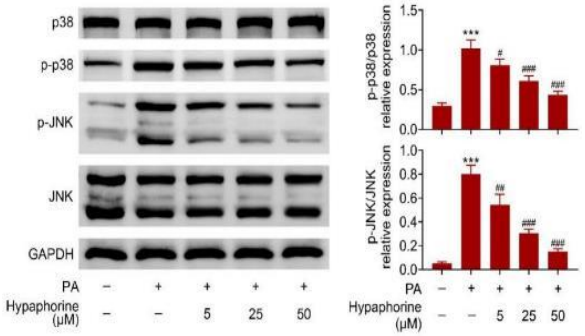

Figure 4: Hypaphorine ameliorates lipid accumulation and inflammation by targeting the p38/JNK and NF-κB pathways. The levels of p-p38 and p-JNK in untreated control AML-12 cells and AML-12 cells treated with PA alone or PA and hypaphorine were detected by immunoblot assay. ***P < 0.001 vs. untreated control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs. PA alone

DISCUSSION

Non-alcoholic fatty liver disease, including both simple fatty liver disease and NASH, is a clinicopathological syndrome characterized by excessive deposition of fat in liver cells [11,12]. The prevalence of NAFLD in the general adult population is 10 – 30 %, of which 10 – 20 % is classified as NASH, and as many as 25 % of patients with NASH develop cirrhosis within 10 years [13]. More effective drugs are therefore needed to combat NAFLD. Hypaphorine, a type of indole alkaloid, may serve as a novel drug for NAFLD treatment.

The results of CCK8 and flow cytometry assays showed that hypaphorine increased the viability of liver cells treated with PA to induce a NAFLD phenotype. Immunoblot assays and ELISA further revealed that hypaphorine suppressed the inflammatory response of PA-treated hepatocytes. In addition, hypaphorine reduced PA-induced lipid accumulation, which was confirmed by Oil red O staining and immunoblot assays. These results suggest that hypaphorine might be beneficial in the treatment of NAFLD.

In a previous study, hypaphorine exerted anti-inflammatory effects in sepsis-induced ALI by targeting the p38/JNK pathway [14]. Similarly, hypaphorine suppressed the inflammatory response in PA-treated hepatocytes through the same pathway. Another study showed that hypaphorine impaired RANKL-induced osteoclastogenesis in mice by suppressing the ERK, p38, JNK, and NF-κB pathways [15]. Furthermore, hypaphorine attenuated PA-induced endothelial inflammation by modulating TLR4 and PPAR-γ, which is dependent on the PI3K/Akt/mTOR pathway. These results suggest that hypaphorine can play multiple roles in the treatment of diseases.

In the present study, the data confirmed that hypaphorine inhibited activation of the p38/JNK pathway in a NAFLD cell model. The p38/JNK pathway mediates inflammatory response and lipid accumulation in various diseases and is critical in NAFLD progression. For example, butorphanol reduced the neuronal inflammatory response and apoptosis by suppressing the p38/JNK/ATF2/p53 pathway [16]; glycyrrhizic acid attenuated the inflammatory response by suppressing high mobility group box-1 (HMGB1) protein via the p38/JNK pathway; EGCG stimulated a pro-inflammatory response in macrophages to suppress bacterial infection by activating the TLR/p38/JNK pathway [17]. These findings suggest that the p38/JNK pathway may serve as a key target in inflammatory diseases. The p38/JNK pathway is also involved in the progression of NAFLD. A previous study indicated that DKK3 expression in hepatocytes influenced susceptibility to liver steatosis and obesity [18]. Further study is needed, however, to uncover the precise mechanism by which the p38/JNK pathway contributes to NAFLD progression.

CONCLUSION

Hypaphorine improves viability suppresses the inflammatory response, and reduces lipid accumulation in PA-treated hepatocytes by targeting p38/JNK and NF-κB pathways. Therefore, hypaphorine may be an effective therapeutic agent for NAFLD. However, *in vivo* studies to validate this finding are required.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and that all liabilities pertaining to claims related to the content of this article will be borne by the authors. Chaoyu Wei and Wei Zhou designed and carried out the study, supervised the data collection, analyzed the data, interpreted the data, prepared the manuscript for publication, and reviewed the drafts of the manuscript.

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