Saturated fatty acid induces cancer stem cell-like properties in human hepatoma cells

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Abstract

Hepatic steatosis has been reported to be a risk factor for the development of liver cancer. The underlying mechanism for carcinogenesis remains to be elucidated. It has been postulated that cancer stem cells (CSCs) within tumor tissues are a subset of cells with stem cell properties of self-renewal and undifferentiation. The purpose of this study was to investigate the effects of a saturated fatty acid, palmitate (PA), on CSC-like properties of human hepatoma HepG2 cells. We investigated the effects of PA on HepG2 cells and primary rat hepatocytes (PRH) by exposing them to PA to induce lipid accumulation. Significant fat accumulation was observed by Oil Red O staining in cells exposed to PA, and it was accompanied by significant increase in NFκB (p65) nuclear translocation in HepG2 cells. Notably, PA significantly enhanced the sphere forming ability of HepG2 cells, but not PRH. Furthermore, PA significantly increased stemness gene expressions of Sox2 and Oct4, and sonic hedgehog (Shh) production. Notably, NFκB inhibitors, N-Acetyl-L-cysteine and pyrollidine dithiocarbamate, and a NOX inhibitor, diphenyleneiodonium, significantly attenuated PA-induced sphere forming ability of HepG2 cells. Our results suggest that lipid accumulation may not only induce pro-inflammatory responses in hepatocytes but may also activate CSC-like properties of hepatoma cells through NFκB activation.

Key words: Palmitate, cancer stem cells, NFκB.

Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) is rising rapidly because of ongoing epidemics of obesity and type 2 diabetes, which become a public health issue. The spectrum of NAFLD starts from simple steatosis to steatohepatitis, liver fibrosis, cirrhosis and finally progresses to hepatocellular carcinoma (1). According to the two-hit-hypothesis of non-alcoholic steatohepatitis (NASH), hepatic steatosis ("first hit") is a prerequisite for subsequent pathologic events ("second hits"), including environmental and genetic factors that result in liver injury (2). Free fatty acids (FFAs) are the major mediators of lipid accumulation in the liver. In NAFLD patients, plasma levels of circulating FFAs are found to be correlated with disease severity (3). Hepatocytes will be damaged when excess FFAs in form of triacylglycerols (TGs) is stored in lipid droplets. Lipid overload will further result in oxidative stress, mitochondrial dysfunction and pro-inflammatory responses ("second hits") (3), leading to inflammation and fibrogenesis in NASH. Recent studies showed that nearly a quarter of NASH patients develop cirrhosis, which is eventually at high risk in developing hepatocellular carcinoma (HCC).

Initiation of cancer may be the result of mutations occurring in stem cells, which interfere with the differentiation of these cells. Many common properties of the stem cells and some tumor cells suggest that cancer stem cells (CSCs) may be responsible for the initiation and progression of cancer. The special property of CSCs is the ability for self-renewal and cell proliferation (spherogenesis), which are the major causes of cancer recurrence and metastasis. Sonic Hedgehog (Shh) signaling pathway and stemness-connected transcription factors, such as Sox2 and Oct4, are primarily responsible for CSC proliferation (4-6). Although the mechanisms of carcinogenesis remains to be clarified, it has been proposed that CSCs may participate in the initiation, progression and metastasis of HCC, and possibly result in the failure of chemo- and radiotherapy. Notably, the sphere formation capacity of cancer cells is a functional approach to enrich the potential CSC subpopulations when the specific markers for CSCs are still controversial (7). Sphere culture in liver cancer was established as a functional approach to isolate, identify and characterize liver CSCs (7).

In this study, we investigated the effects of a saturated fatty acid, palmitate (PA), on CSC-like properties in HepG2 cells. Our results demonstrated that exposure of hepatocytes to PA resulted in NFκB activation and inflammatory cytokine expression in HepG2 cells. Furthermore, PA significantly enhanced the sphere forming ability associated with the significant increase of stemness gene expressions of Sox2 and Oct4, and Shh production in HepG2 cells. Notably, NFκB inhibitors, N-Acetyl-L-cysteine (NAC), and pyrollidine dithiocarbamate (PDTC), and a NADPH oxidase (NOX) inhibitor, diphenyleneiodonium (DPI), could significantly reduce PA-induced sphere forming ability of HepG2 cells. Our results suggest that lipid accumulation not only induced NFκB activation, but also activate CSC-like properties of HepG2. This study elicits the possibility that saturated fatty acid, such as PA, might activate CSC-like pro-

perties of hepatoma cells in NAFLD patients through NFκB pathway. Thus, this study provides a promising model to study the mechanisms of carcinogenesis in NAFLD and to develop chemopreventive therapies for NAFLD.

Materials and methods

Cell culture (human HepG2 cells and primary rat hepatocyte, PRH)

The human hepatoma cell line, HepG2, was grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal calf serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2. The HepG2 cells were passaged in the T75 flask and maintained in DMEM containing 10% FBS.

Primary hepatocyte isolation and culture

Hepatocytes were isolated from male Wistar rats (275-300 g) using a two-stage non-recirculating perfusion method described previously (8, 9). Livers were perfused for 10 min via the portal vein with Ca^{2+}-, Mg^{2+}-free Herb's buffer, containing 1% antibiotics, followed by buffered with antibiotics and 0.25 mg/ml collagenase Type IV (435 U/mg; Sigma; MO, USA) for at least 7 minutes. The minced liver was passed through two layers of cheesecloth and hepatocytes were re-suspended in Herb's buffer and filtered through nylon (62 mesh). After centrifugation for 5 min at 100 x g, hepatocytes were re-suspended in DMEM and filtered through two layers of nylon. Hepatocytes were added to 6-well plates coated with collagen film (Vitrogen 100; Collagen Corp., Palo Alto, CA, USA) at 2×10^5 cells/well density. After 4 hours, unattached cells were removed and fresh growth medium was applied. During 20 h incubation, cells were treated with PA for indicated time and concentration as described.

Chemicals

Palmitic acid, albumin (BSA), N-Acetyl-L-cysteine (NAC), pyrollidine dithiocarbamate (PTDC) and diphenyleneiodonium (DPI) were purchased from Sigma-Aldrich. Palmitate (PA; 102553; Sigma) stock solution was carried out as described previously (10, 11). Briefly, a 400 mM PA stock solution was prepared in DMSO. A filtered 3% (w/v) FFA-free BSA (9048-46-8; Sigma) solution was prepared in DMEM and maintained at 55 °C in a water bath. 4 mM FFA/BSA solution was obtained by mixing the appropriate amount of PA stock solution to 3% BSA at 55 °C for another 30 min. FFA/BSA solution was diluted with DMEM and adjusted to the concentration for treatment. The above solution was fresh prepared before use. The 62.5 mM NAC (A9165; Sigma) stock solution, 50 mM PDTC (P8765; Sigma) stock solution and 5 mM DPI (D2926; Sigma) stock solution were prepared in ddH_2O and stored in -20°C.

Oil Red O Staining

Sub-confluent monolayers of HepG2 cells were exposed to PA or BSA for 12 hours. Cells were stained with Oil-Red-O to examine the amount of fat accumulation in the cells. Briefly, dishes were washed with cold phosphate-buffered saline and fixed in 10% neutral formalin. After 2 changes of propylene glycol, Oil-Red-O was added with agitation for 7 minutes, followed by washing in 85% propylene glycol. The dishes were then rinsed in distilled water and counterstained with hematoxylin. For each dish, three images were photographed, and a representative image is shown.

Transiently transfected cells and luciferase assays

Cell were seeded at a density of 10^5 cells/well on 24-well plates the day before transfection. Plasmid NFκB-Luc (1 μg/well) (Strategene, La Jolla, CA, USA) and pRL-SV40 (0.2 μg/well) (Promega, Madison, USA) were transfected into cells by lipofectamine (Invitrogen, California, USA). The pNFκB-Luc consists of NFκB-binding region, followed by the reporter gene firefly luciferase. Plasmid pRL-SV40 served as an internal control to normalize the transfection efficiency (10, 11). After treatment with PA for 6 h in a 5% CO_2 incubator at 37 °C, the cells were harvested and lysed in 100 μL of lysis reagent. Twenty mircoliter of cell lysate was then mixed with 100 μL of luciferin (the substrate of luciferase) right before luminescence detection. The luminescence, generated by luciferase activity, was measured with an AutoLumat LB953 (Berthold Technologies, Bad Wildbad, Germany). All reagents for luciferase assays were purchased from Promega (Madison, USA).

Western blotting analyses for Shh, NFκB (p65) nuclear translocation

Cells (5×10^6) were seeded in medium containing 10% FBS. After 24 h, the cells were washed twice with PBS and the medium was replaced by serum-free medium. PRH or HepG2 cells (in serum-free medium) were treated with PA for 6 h in 5% CO_2 incubator at 37 °C. After treatments, nuclear extracts containing the NFκB active protein were prepared from cells using a nuclear extraction kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions and our published methods (10, 11). Twenty microgram of proteins in cytoplasmic fraction or nuclear fraction was separated on a 10% SDS-PAGE and transferred onto Immobilon-PVDF (Millipore, Bedford, MA, USA) in a transfer buffer (6.2 mM boric acid, pH8.0). The blots were incubated initially with blocking buffer (5% BSA) for 1 h at room temperature, and then with specific primary antibodies against α-actin (GTX100095, GeneTex, Taiwan), α-tubulin (sc-8035, Santa Cruz), PCNA (sc-56, Santa Cruz) or NFκB (p65) (MAB3026, Santa Cruz) and Shh (MABD175, Millipore). Primary antibodies had been diluted (1:10000) with Tris-buffered saline-Tween 20 (TBS-T) containing 1% BSA. After antibody incubation, the blots were washed with TBS-T and incubated with HRP conjugated secondary antibodies (1:2000) (Santa Cruz) for 1 h at room temperature. After washing the secondary antibodies with TBS-T, immune-detection was performed by Luminescence Imaging System (FUJI las-4000mini, Japan). Film exposure ranged from a few seconds to 5 minutes. Bands were quantified using Kodak Image Analysis Software (Rochester, NY, USA).

Semi-quantitative and quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Semi-quantitative (for ICAM1, IL8, TNF-α, Shh) and quantitative RT-PCR (for Sox2, Oct4, Nanog) as-

says were employed to assess the relative levels of the mRNA expression of PA-stimulated HepG2 cells and PRH (10-12). Total RNA was isolated from HepG2 cells or PRH by the method of Chomczynski and Sacchi (13). For cDNA synthesis, 1 mg of total RNA was reverse-transcribed in a 30 ml of reaction mixture containing 10 mM dNTP mix, 500 μg/ml oligo(dT), 0.2 mM dithiothreitol, 40 units of RNase inhibitor, 200 units of M-MLV reverse transcriptase, and 5x buffer (with 1.5 mM $MgCl_2$) (Invitrogen, USA). The reaction mixture was incubated at 37 °C for 60 minutes and then denatured at 65 °C for 10 minutes. Quantitative PCR analysis was performed using an ABI prism 7900 HT Sequence Detection System (Applied Biosystems). The specific primers used in this study were described in Table 1.

Sphere formation assay

PRH or HepG2 cells were maintained as a monolayer in high glucose DMEM with 10% FBS, 100-IU/ml penicillin G and 100-μg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. Cells were collected and washed to remove serum, then suspended in serum-free DMEM/F12 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, Dulbecco's modified Eagle's medium HG/F-12 supplemented with B27 (Invitrogen), 50 ng/ml epidermal growth factor, 20 ng/ml FGF2, and 5 μg/ml heparin, 2% B27, 1% N2 supplement (Invitrogen, Carlsbad, CA, USA). The cells were subsequently cultured in ultra low attachment 6 cm dishes (Corning Inc., Corning, NY, USA) at a density of no more than 1x 10^4 cells/well in 5 ml sphere medium. Cells were cultured in 5% CO_2 in a 37 °C incubator. The spheres (diameter between 70-150 μm and larger than 150 μm) were both counted directly under microscope after 7 days *in vitro* (7). Due to the fact that cell aggregation may affect the numbers of spheres. One large

sphere may be due to the aggregation of several small spheres. Therefore, we investigated the effects of PA on the sphere formation of HepG2 cells by counting the numbers of spheres in two different sizes.

Data analysis

Data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was used for comparison of biochemical and molecular parameters. A non-parametric method (the Dunn procedure under the Kruskal–Wallis test) was used for multiple pairwise comparisons between groups for the histological grades of fibrosis. Statistical significance was accepted at $p < 0.05$.

Results

Effects of PA on NFκB (p65) nuclear translocation and NFκB luciferase activity in HepG2 cells

We have previously demonstrated that lipid accumulation in the liver led to hepatic inflammation and fibrogenesis through NFκB pathway (12). To clarify if NFκB-mediated hepatic inflammation contributes to any effect on cancerous cells in NASH patients, we investigated the effects of saturated fatty acid PA on NFκB (p65) nuclear translocation and NFκB luciferase activity in HepG2 cells. The results showed that PA (200 μM) induced NFκB (p65) nuclear translocation and NFκB luciferase activity in HepG2 cells significantly (Figures 1a, *$p<0.05$). Interestingly, PA (200 μM)-induced NFκB (p65) nuclear translocation was also regulated in a time-dependent manner, reaching a maximum at 6 h after PA treatment (Figure 1b, **$p<0.01$). The functional effect of PA on NFκB activity was further examined by the lu-

Table 1.

Target genes	Primer Sequences for Human Genes
GAPDH	Forward: 5'-GAAGGTGAAGGTCGGAGTC-3' Reverse: 5'-GAAGATGGTGATGGGATTTC3'
IL-8	Forward: 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' Reverse: 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'
ICAM-1	Forward: 5'-GGCTGGAGCTGTTTGAGAAC-3' Reverse: 5'-TCACACTGACTGAGGCCTTG-3'
Shh	Forward: 5'-GGCTGGAGCTGTTTGAGAAC-3' Reverse: 5'-TCACACTGACTGAGGCCTTG-3'
TNF-α	Forward: 5'-CCTGTGAGGAGGACGAACAT-3' Reverse: 5'-GAGGAAGGCCTAAGGTCCAC-3'
hSox2	Forward: 5'- CGCAGACCTACATGAACG-3' Reverse: 5'- CCCTGGAGTGGGAGGAA-3'
hOct4	Forward: 5'- AAAGCAGAAACCCTCGT-3' Reverse: 5'- TCCAGGTTGCCTCTCAC-3'
hNanog	Forward: 5'- GAGACAGAAATACCTCAGCC-3' Reverse: 5'- TCTGCGTCACACCATTG-3'

| Target genes	Primer Sequences for Rat Genes
rGAPDH	Forward: 5'-AGCCCAGAACATCATCCCTG-3' Reverse: 5'-CACCACCTTCTTGATGTCATC-3'
rIL-8	Forward: 5'-AGGGATTCACTTCAAGAACA-3' Reverse: 5'-GCATCTTTTGGACAATCTTC-3'
rICAM-1	Forward: 5'-CACTAGAGGAGTGAGCAGGTTAACAT-3' Reverse: 5'-TATGACTCGTGAAAGAAATCAGCTCTT-3'
rTNF-α	Forward: 5'-GCCAATGGCATGGATCTCAAAG-3' Reverse: 5'-CAGAGCAATGACTCCAAAGT-3'
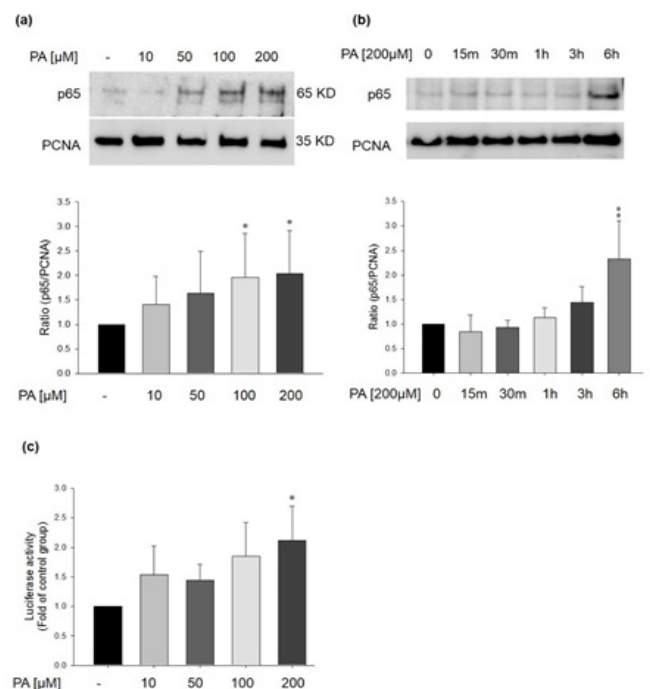
Figure 1. Effects of PA on NFκB (p65) nuclear translocation and promoter activity of HepG2 cells. (a) PA [10-200 μM] concentration-dependently increased NFκB (p65) nuclear translocation in HepG2 cells. PA [200 μM] significantly increased NFκB (p65) nuclear translocation in HepG2 cells (n=3). **p<0.01 vs. control. (b) PA [200 μM] time-dependently increased NFκB (p65) nuclear translocation in HepG2 cells. (c) PA [200 μM] significantly increased NFκB promoter activity in HepG2 cells (n=3). *p<0.05 vs. control.
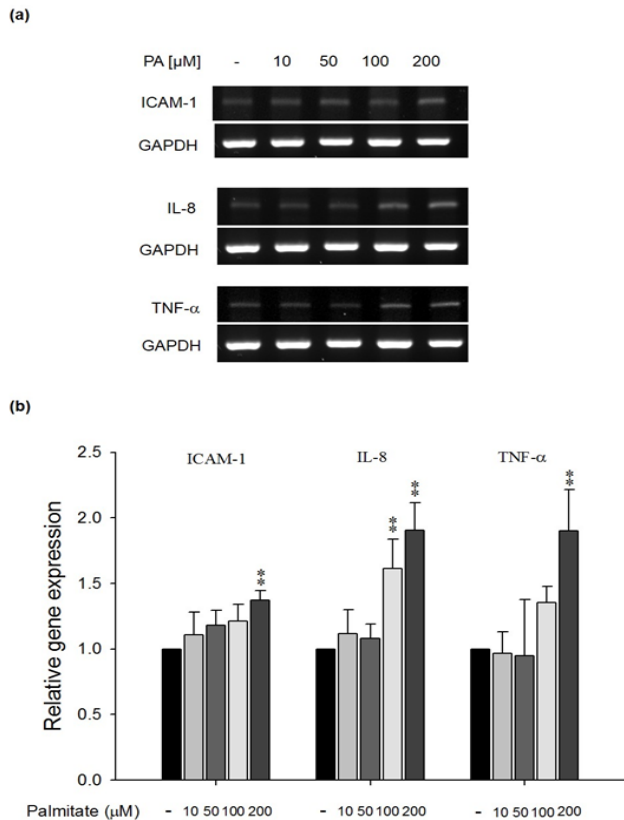
(a)

(b)

Figure 2. Effects of palmitate on mRNA expressions of proin-flammatory genes (ICAM-1, IL-8, TNF-α) in HepG2 cells. (a, b) PA treatment significantly induced the mRNA expressions of *ICAM-1*, *IL-8*, *TNF-α* genes in HepG2 cells (n=3). *p<0.05 vs. control; **p<0.01 vs. control.

ciferase activity assay. PA induced the NFκB-mediated promoter activity in a dose-dependent manner (Figure 1c). Taken together, the results suggest that PA could induce translocation of NFκB (p65) into nucleus, leading to transcriptional activation of the downstream genes of NFκB in regulation of cellular functions.

Effects of PA on mRNA expression of pro-inflammatory genes (ICAM-1, IL-8 and TNF-α) in HepG2 cells

To clarify if NFκB-mediated pro-inflammatory cyto-kines contributes to any effect on cancerous cells pa-tients, we investigated the effects of saturated fatty acid PA on mRNA expression of pro-inflammatory genes (ICAM-1, IL-8 and TNF-α) in HepG2 cells. PA (200 μM) significantly stimulated the mRNA expression levels of *ICAM-1*, *IL-8* and *TNF-α* genes in HepG2 cells (Figures 2a and 2b), consistently suggesting that lipid accumulation might induce inflammatory responses.

Effects of PA on the expression of stemness genes (Oct4, Sox2, Nanog and Shh) in HepG2 cells

To clarify if PA contributes to any effect on cancerous cells, we further investigated the effects of PA on the expression of stemness genes (Oct4, Sox2, Nanog and Shh) in HepG2 cells. PA treatment significantly induced the mRNA expression of stemness genes, such as *Sox2* (Figure 3a, n=3, **p<0.01) and *Oct4* (Figure 3b, n=3, *p<0.05, **p<0.01). Protein and mRNA expressions of Shh were also both up-regulated in PA-treated HepG2 cells (Figures 3d and 3e, n=3, *p<0.05, **p<0.01, respectively). In contrast, PA treatment did not alter the mRNA expression of Nanog (Figure 3c, n=3). The data suggested that PA might potentially induced self-renewal and tumor-initiation CSC-like properties of HCC cells.

Effects of PA on sphere formation abilities of HepG2 cells, but not normal PRH

We hypothesized that PA could enhance CSC-like property in cancerous cells (HepG2) rather than normal cells (PRH). Therefore, we compared the sphere formation ability between these two cell lines. The results showed that PRH did not have sphere formation after PA (200 μM) treatment (Figure 4a). However, PA (200 μM) significantly enhanced the sphere formation of HepG2 cells (Figure 4a). Lipid accumulation was observed in HepG2 cells by exposing them to PA (200 μM) to simulate the excessive influx of fatty acids into cells. Significant fat accumulation was observed by Oil Red O staining in cells exposed to PA (Figure 4b). PA could induce significantly more spheres of HepG2 cells (Figure 4c, n=4, **p<0.01). Furthermore, NAC, PDTC
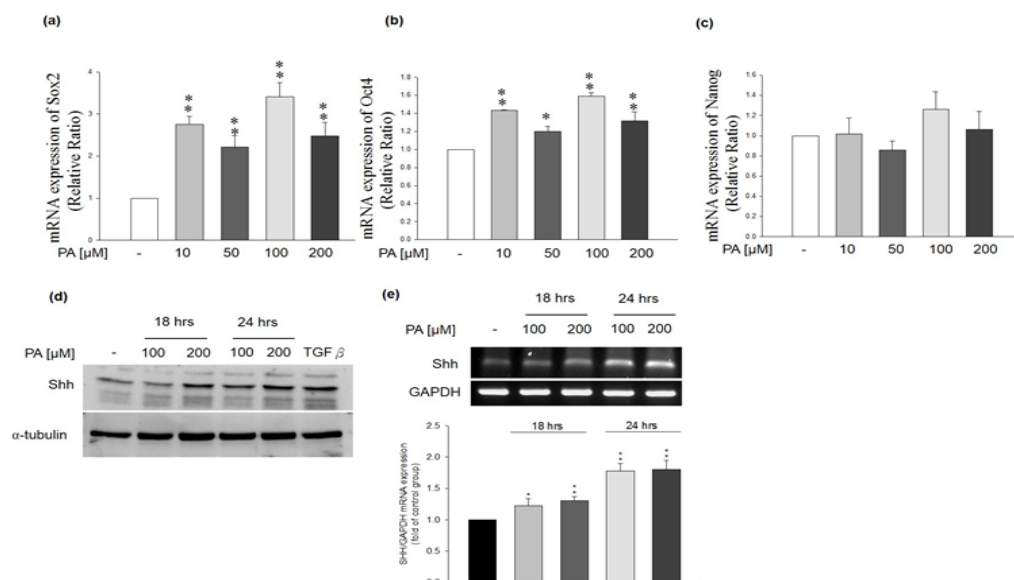

Figure 3. Effects of PA on stemness gene expression in HepG2 cells. PA treatment significantly induced the mRNA expression of (a) *Sox2* (n=3). **p<0.01, (b) *Oct4* (n=3). *p<0.05, **p<0.01. (c) PA treatment did not alter the mRNA expression of *Nanog*. (d) PA treatment significantly induced the protein production of Shh. (e) PA treatment significantly induced the mRNA expression level of *Shh* (n=3, *p<0.05, **p<0.01).
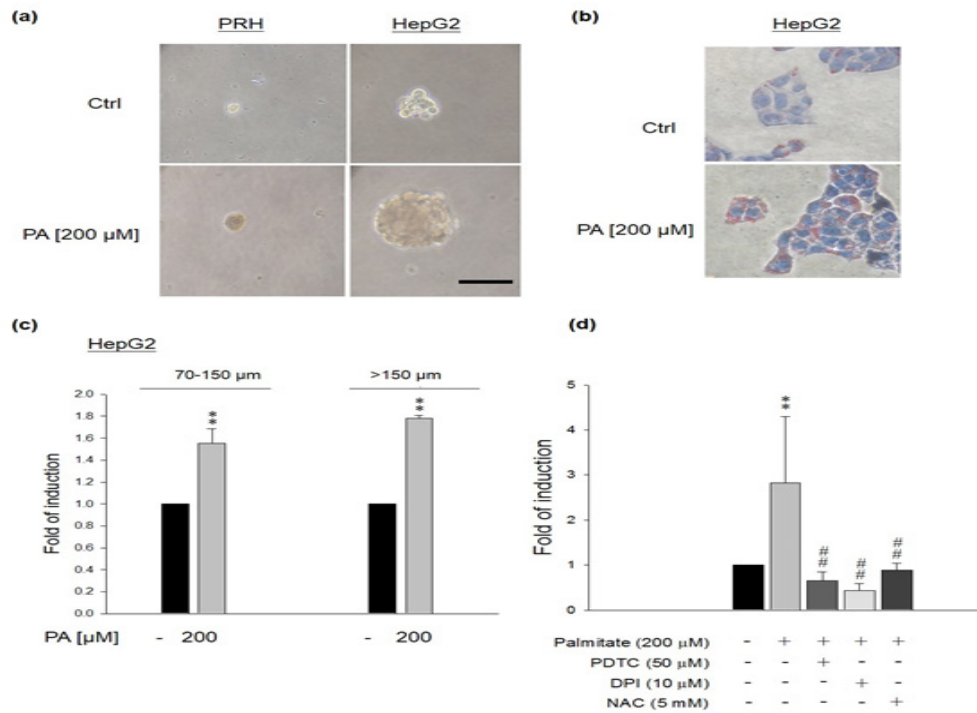
Figure 4. Effects of PA on sphere formation in PRH and HepG2 cells. (a) PA (200 μM) significantly enhanced the sphere formation of HepG2 cells, but not PRH. (b) Lipid accumulation was observed in HepG2 cells by exposing them to PA (200 μM) to stimulate the excessive influx of fatty acids into cells. Significant fat accumulation was observed by Oil Red O staining in cells exposed to PA. (c) PA could induce significantly more spheres in HepG2 cells (n=4, **$p<0.01$). (d) NAC, PDTC and DPI could attenuate palmitate-induced sphere formation in HepG2 cells (n=4, **$p<0.01$ vs. control, ##$p<0.01$ vs. PA treatment).

and DPI could attenuate PA-induced sphere formation in HepG2 cells (Figure 4d), indicating that PA could induce the sphere formation of HepG2 cells through NFκB and NOX activation.

Discussion

In this study, we demonstrated a potential mechanism for the pathophysiological role of hepatic lipid accumulation during liver carcinogenesis. Our previous study demonstrated that PRH and HepG2 cells incubated with PA showed increased ROS production, NOX expression (12). Conditioned media from PRH treated with PA led to the activation of HSC-T6 cells and fibrogenesis (12). In this study, we showed that PA significantly induced the activation of NFκB signaling pathway, such as NFκB (p65) nuclear translocation and promoter activity (Figure 1), and mRNA expression of proinflammatory genes (Figure 2) in HepG2 cells. In particular, PA significantly enhanced stemness gene expression and Shh production in HepG2 cells (Figures 3). Moreover, PA also significantly increased sphere formation in HepG2 cells, which could be inhibited by NFκB and NOX inhibitors (Figure 4). In our previous study, increased ROS formation was observed in PA-treated PRH and HepG2 cells (12). Oxidative stress (OS) causes lipid peroxidation in cell membranes. Lipid peroxidation products can induce inflammatory responses with up-regulation of pro-inflammatory cytokines (14). Our results demonstrated increased *ICAM-1*, *IL-8* and *TNF-α* gene transcripts in HepG2 cells after PA treatment (Figure 2). The elevated pro-inflammatory gene expression could be a result of OS induced by lipid accumulation (15). PA has also been demonstrated to induce IL-8 production from hepatocytes and HepG2 cells (16).

Saturated free fatty acids have been suggested to play important roles in many biological processes such as hormone production and initiation of signaling pathways downstream the cellular membrane (17). Palmitoylation is one of the most important posttranslational modifications in cellular proteins that is mediated by a membrane-bound palmitoyl acyl transferase (17). Palmitoylation has been shown to enhance the hydrophobicity and membrane affinity of the target protein. It also involves in regulating cytosolic proteins' trafficking, stability and sorting. Dysregulation of palmitoylation process has been shown in different cancer types such as colon cancer (18), prostate cancer (19) and breast cancer (20, 21). The Wnt and Hedgehog (Hh) pathways have been suggested their importance in embryonic development and the maintenance of CSCs (22). Hh ligand overexpression is correlated with HCC (23), pancreatic (24) and breast cancers (25). Notably, palmitoylation of Wnt and Hh determines their signaling activity (22). Furthermore, palmitoylation of Wnt is critical for the binding capacity to its Frizzled receptors in cell surface and induce downstream stabilization of β-catenin (26). In addition, palmitoylation of Hh facilitates its binding to the cell surface, which mediates its interaction with the receptor Patched to activate the downstream Gli transcription factors (27). In this study, our data showed that PA treatment induced the mRNA and protein expression of Shh in HepG2 cells (Figure 3). Future efforts in identifying the palmitoylated proteins in PA-treated HepG2 cells will help elucidate the mechanism of PA-induced CSC properties.

Recently, the sphere formation capacity of cancer cells has been demonstrated to enrich the potential CSC populations. Sphere culture in liver cancer was also established as an functional approach to isolate, identify and characterize liver CSCs (7). In this study, we observed that PA significantly increased the numbers

of spheres in HepG2 cells, but not in PRH (Figure 4a). This phenomenon suggested that lipid accumulation might enhance the CSC-like properties, such as Sox2 (Figure 3a), Oct4 (Figure 3b), Shh expressions (Figure 3d and 3e) and sphere forming ability (Figures 4a and 4c) in HepG2 cells. Not surprisingly, PRH has no CSC properties and could not form spheres under sphere culture conditions (Figure 4a). On the other hand, the human hepatoma HepG2 cells showed enhanced CSC-like properties in the presence of PA. In particular, the positive correlation between NFκB (p65) and Shh expression has been demonstrated in the dissected specimens of chronic pancreatitis and pancreatic adeno-carcinoma (24). In pancreatic cancer cells, suppression of NFκB could downregulate the mRNA expression of Shh (24). Further activation of NFκB by inflammatory stimuli (IL-1β, TNF-α, and lipopolysaccharide) can induce the upregulation of Shh, further resulting in activation of the Hh pathway. Notably, overexpression of Shh induced by these stimuli can also be suppressed by blockade of NFκB. Therefore, NFκB-mediated Shh expression results in the activation of the Hh pathway and enhanced cell proliferation in cancer cells (24).

Sox2 is the key transcription factor essential to pluripotency and is demonstrated its crucial role in many types of cancers. Metastasis and low survival rate in HCC have been correlated with expression of Sox2. Therefore, Sox2 plays an important role in HCC prognosis and treatment (5). In addition, co-expression of Sox2 and Oct4 in HCC has been suggested the aggressive tumor characteristics and a worse clinical outcome (6). Notably, the in situ expression of Sox2 and Oct4 mRNA were both found in hepatoma cell lines and tumor tissues. Therefore, the expression of Sox2 and Oct4 can be utilized as novel predictors of poor prognosis for patients undergoing resection of HCC (4). Knockdown of Sox2 and Oct4 gene expression in HCC cells can reduce the sphere formation and increase cellular sensitivity to chemotherapy, indicating that Sox2 and/or Oct4 may be a promising target in clinical cancer treatment (16). To our knowledge, we are the first to show the effects of PA on CSC-like properties (spherogenesis and stemness marker expression) of hepatoma cells, we also demonstrate that NFκB not only played an important role in PA-mediated pro-inflammatory (17) and profibrogenic effects (9), but also regulated the CSC-like properties in HepG2 cells. Using choline-deficient diet to induce the accumulation of lipid droplets in rat liver, we also observed both pro-inflammatory and pro-fibrogenic responses by lipid accumulation. Notably, NFκB activation and GSTP expression, which is a preneoplastic marker, were both elevated in the liver (data not shown), suggesting the *in vivo* effects of lipid accumulation on the progression of liver cancer.

It has been shown that the epithelial ovarian CSCs also have NFκB-mediated stemness properties and represent the chemo-resistant population (28). Our results are in agreement with their findings and suggest a possible application of NFκB inhibitors in the treatment of NASH-mediated carcinogenesis (29). As a proof of principle, curcumin has recently been shown to induce the cell death, down-regulation of CSC markers and suppressed tumorigenicity of CSCs in HCC cell lines through NFκB inhibition (30). In summary, our results

suggested that lipid accumulation might not only induce ROS production and pro-inflammatory responses in hepatocytes (12) but also activated CSC-like properties of hepatoma cells. This study suggests the possibility that saturated fatty acid might activate CSC-like properties of hepatoma cells in NAFLD patients. Thus, this study provides an attractive model to study the mechanisms of carcinogenesis in NAFLD and to identify and test novel therapies for chemopreventive therapies in NAFLD.

Acknowledgements

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