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Original Research Articles

Free Fatty Acid Induces Apoptosis via Retinoic Acid Receptor-Related Orphan Receptor Alpha (RORA)-Mediated Protein Tyrosine Phosphatase Non-Receptor Type 2 (PTPN2) Downregulation in Hepatic Cells

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Abstract: Background: Retinoic Acid Receptor-Related Orphan Receptor Alpha (RORA) is a transcription factor in addition to a nuclear orphan receptor. It was shown to regulate the expression of various proteins that regulate hepatic functions. RORA expression was downregulated under Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) conditions; however, there is limited data on the mechanisms by which RORA regulates MASLD. Protein Tyrosine Phosphatase Non-Receptor Type 2 (PTPN2) participates in the process of dephosphorylating various receptor and non-receptor tyrosine protein kinases, which play a significant role in the signalling of various cellular operations. PTPN2 was reported to be suppressed in obesity-associated metabolic dysfunction-associated steatohepatitis (MASH). **Methods:** HepG2 cells were treated with 300 µM palmitic acid (PA) for 72 h, and the expression of RORA, PTPN2, Bcl-xL (BCL2L1), BAX, and β-actin was determined using immunoblotting. Promoter analysis of PTPN2 was performed through JASPAR CORE database enrichment analysis. HepG2 cells were treated with either RORA natural agonist or synthetic antagonist, and the regulation of PTPN2 and apoptosis was studied. siRNAs targeting PTPN2 (siPTPN2) were transfected into HepG2 cells, and PTPN2 and apoptotic marker expression was assessed. Three sets of six C57BL/6 mice were given either CSAA, CDAA, or a normal diet for a total of 54 weeks. Liver tissues were separated after feeding, and immunoblotting was performed using total protein. **Key Findings:** RORA and PTPN2 expression were downregulated, and apoptosis was upregulated in PA-treated hepatic cells. JASPAR CORE database enrichment analysis of PTPN2 promoter analysis revealed the presence of ROR response element (RORE). PTPN2 expression was upregulated and downregulated in hepatic cells treated with RORA natural agonist and synthetic antagonist, respectively. Apoptosis was unregulated in siPTPN2-transfected hepatic cells. In CDAA diet-fed mice, RORA and PTPN2 expression were suppressed, and apoptotic markers were upregulated. **Conclusions:** According to the study's findings, palmitic acid induced apoptosis by inhibiting RORA-mediated PTPN2 expression in hepatic cells. These findings can help in the development of novel therapeutic modalities using potent activators of RORA for the treatment of MASLD.

Keywords: RORA; PTPN2; apoptosis; CDAA; palmitic acid

1. Introduction

The liver is the focal point for various physiological functions. It performs multiple functions, including carbohydrate metabolism, lipid and cholesterol homeostasis, the endocrine function of glycemic control, the exocrine function of bile production, regulation of blood volume, immunity, vitamin and mineral storage, and detoxification of drugs and xenobiotic compounds [1]. It is affected by various agents like hepatitis virus, fat overload, and chronic alcoholism, which lead to chronic liver disease and further hepatocellular carcinoma (HCC) [2]. Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) represents a notable spectrum of severe liver complications, including steatosis, fibrosis, cirrhosis, and HCC [3].

Fat accumulation during MASLD is the prime trigger for inflammation and oxidative stress [4]. Due to this lipotoxicity, the hepatic cells are directed toward apoptosis in later stages. Apoptosis is a biological safeguard mechanism that triggers the cell to undergo programmed cell death upon various stimuli. It includes intrinsic and extrinsic pathways based on the origin of the stimulus [5]. Palmitic Acid (PA), a saturated free fatty acid (FFA), was reported to be elevated in the serum of MASLD patients [6]. PA-mediated lipotoxicity is a crucial player in MASLD progression [7]. PA was shown to activate the intrinsic apoptotic pathway [8]. The protein family B cell lymphoma 2 (BCL-2) was primarily responsible for controlling this pathway.

Nuclear receptors, a class of transcription factors, have been a significant target for treating MASLD due to their control of multiple gene expressions involving various functions [9]. Retinoic Acid Receptor-Related Orphan Receptor Alpha (RORA), a nuclear receptor family member, has been under investigation in recent years due to its involvement in various hepatic functions. Recent studies reported that RORA is suppressed in the MASLD spectrum, and its activation reversed the MASLD progression [10–13].

Protein Tyrosine Phosphatase Non-Receptor Type 2 (PTPN2), also commonly called T-Cell Protein Tyrosine Phosphatase (TCPTP), is a tyrosine phosphatase and controls cellular functions by dephosphorylating receptor and non-receptor protein tyrosine kinases like Janus Kinase-Signal Transducer And Activator Of Transcription (JAK-STAT) proteins and Src family kinases [14]. Recent reports suggested PTPN2 deficiency in hepatic cells enhanced the progression of MASLD towards HCC [15]. In this study, we showed that RORA inhibited apoptosis by upregulating PTPN2 expression. The addition of PA reversed this process by inhibiting the expression of RORA in hepatic cells.

2. Materials and Methods

2.1. Cell Culture and Treatment

HepG2 cells were maintained in complete growth media consisting of Dulbecco's Modified Eagle Medium (DMEM; HiMedia Laboratories, Mumbai, India. Cat No. AL007G) complemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, New York, USA. Cat No. A5256701) and 1% antibiotic (penicillin-streptomycin (10,000 U/mL); Thermo Fisher Scientific, Cat No. 15140148). HepG2 cells were plated at around 4 × 105 cells/well on a 6-well plate (Eppendorf, Hamburg, Germany. Cat. No. 0030720113). After 24 h, the cells were incubated with palmitic acid (PA; 300 μM; Sigma, Missouri, USA. Cat No. P5585) conjugated with bovine serum albumin (MP Biologicals, Santa Ana, CA, USA, Cat No. 0216006980) in 6:1 molar ratio. After 72 h incubation, cells were collected for protein isolation. Similarly, the cells were treated with either DMSO (HiMedia Laboratories, Cat No. TC185) or RORA natural agonist, namely, Cholesterol sulphate (CS) (Cayman, Michigan, USA. Cat No. 15106) or RORA synthetic antagonist, namely, SR3335 (Cayman, Cat No. 12072). Post-treatment, cells were incubated for 24 h.

2.2. CDAA-Induced MASLD Mice Model System

Prior approval for all the in vivo experiments was acquired from the Animal Ethics Committee of South Asian University, New Delhi, India. Five to six-week-old male C57BL/6 mice (Hylasco Bio-Technology Pvt. Ltd., Hyderabad, India) were collated into three groups (6 mice per group) and further fed with a normal chow diet (standard diet), choline sufficient L-amino acid defined (CSAA) diet (Dyets Inc., Pennsylvania, USA. Cat. No. 518754), or choline-deficient L-amino acid defined (CDAA) diet (Dyets Inc., Cat. No. 518753) diet for 54 weeks [16]. After feeding, mice were euthanized, and their livers were collected post-dissection. The liver tissues were promptly snap-frozen after being cleaned with PBS and kept at −80 °C until they were utilized for the total protein isolation process.

2.3. Transfection Experiments

Either siRNAs against PTPN2 (siPTPN2) (Genscript, Piscataway, NJ, USA) or scrambled RNA (NS) (Dharmacon, Horizon Discovery, Waterbeach, UK, Cat No. CN-001000-01) were transfected in HepG2 cells using siPORT NeoFX transfection reagent (Invitrogen, Thermo Fisher Scientific, Cat No. AM4510) as per the manufacturer's protocol. After 72 h, the cells were used to isolate the protein.

2.4. Western Blotting

Six-well plates containing HepG2 cells were added with lysis buffer containing mammalian protein extraction reagent (MPER; Thermo Fisher Scientific, Cat No. 78503) supplemented with protease inhibitor cocktail (PIC; 100:1 ratio; Thermo Fisher Scientific, Cat No. 87786), then incubated on ice for ten minutes. The cells were collected using a cell scraper and sonicated. The cleared lysate was centrifuged at 10,000× *g* for 10 min at 4 °C, and the protein supernatant was collected. With the bicinchoninic acid protein test kit (Pierce, Thermo Fisher Scientific, Cat No. A65453), the amount of total protein was measured, and a 12% SDS-PAGE gel was used to resolve total protein in an equal amount (40 µg). Resolved protein was then transferred onto polyvinylidene fluoride (PVDF) membranes, and post-transfer, the membranes were blocked with 5% skim milk (HiMedia Laboratories, Cat. No. GRM1254) for 1 h, followed by incubating overnight at 4 °C with primary antibodies against RORA (CST#34639), TCPTP (PTPN2) (CST #58935), Bcl-xL (BCL2L1) (CST #2764), BAX (CST #2772), or β-actin (Sigma #A5441). Tris-buffered saline containing 0.1% Tween 20 (TBST) was used to wash the membranes before HRP-conjugated specific secondary antibodies [anti-rabbit (Invitrogen #31462) or anti-mouse (Invitrogen #31432)] were added. Following a one-hour incubation period at room temperature, the membranes were washed and imaged in a Bio-Rad Chemidoc imager using Clarity Western ECL Substrate (Bio-Rad, California, USA. Cat. No. 170-5060). Using Image J software (version 1.54p; National Institutes of Health, USA), the band intensities were quantified for analysis.

2.5. Statistical Analysis

Three replicates of each in vitro experiment were conducted. A total of 6 mouse samples per group were taken for in vivo mouse data. Mean and standard deviation were calculated for both in vitro experiments and the mouse sample groups. The significant difference between the two groups was determined using the unpaired Student's t-test, and a p-value of less than 0.05 ($p \leq 0.05$) was regarded as significant.

3. Results

3.1. PA Downregulates PTPN2 and Triggers Apoptosis

HepG2 cells were incubated with PA (300 µM) for 72 h. Every 24 h, the change of PA and fresh media was performed. Post-incubation, cells were collected, and total protein was isolated as stated in the methods. Western blot results showed that PA significantly decreased the expression of PTPN2 and anti-apoptotic protein Bcl-xL in HepG2 cells, while it significantly increased the pro-apoptotic protein BAX expression (Figure 1a). Following quantification and plotting of band intensities, the data showed that there was a two-fold downregulation of PTPN2 and Bcl-xL, whereas expression of BAX was upregulated by two-fold (Figure 1b).

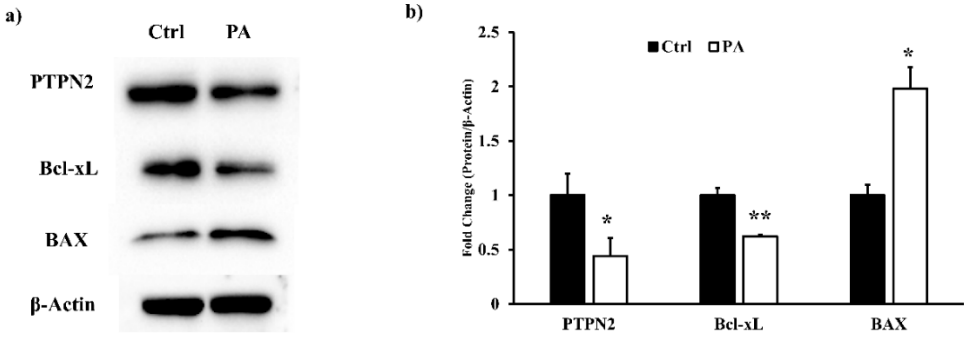

Figure 1. PA downregulates PTPN2 and upregulates apoptosis in hepatic cells. (**a**) HepG2 cells were cultured and incubated with Palmitic Acid (PA; 300 µM) for 72 h, and total cellular protein was isolated for Western blot analysis. The PA treatment suppressed the expression of PTPN2 and triggered apoptosis. (**b**) The band intensities were quantified and represented as fold change (protein/b-actin) (n = 3; * $p \leq 0.05$, ** $p \leq 0.01$).

3.2. PTPN2 Promoter Contains RORE Elements and RORA Is Downregulated in PA Treated Cells

The promoter of PTPN2 was further analyzed to observe its controlling elements. The promoter sequence of human PTPN2 has been retrieved from the Eukaryotic Promoter Database (EPD—https://epd.expasy.org/epd/) in the range from −1000 to 0 bp relative to the Transcription Start Site (TSS). The retrieved promoter was scanned in the JASPAR database for nuclear receptors with C4 zinc fingers class-based transcription factor binding sites with a relative profile score threshold of 95%. We observed that NR2C1, NR2C2, RORC, RORA, and RORB nuclear receptors showed higher relative scores. Out of them, RORA had the highest score (Figure 2a). To cross-validate these results, the promoter of PTPN2 was scanned on CiiiDER [17], a tool for predicting and analysing transcription factor binding sites. The tool yielded similar results as JASPAR database scanning results, revealing RORA transcription factor binding sites (ROR response element (RORE)) in the PTPN2 promoter region (Figure 2b).

To check the relative expression of RORA in PA-treated hepatic cells, HepG2 cells were incubated with PA (300 µM) for 72 h. Every 24 h, the change of PA and fresh media was performed. Post-incubation, cells were collected, and total protein was isolated as stated in the methods. As per the results of the Western blot, the expression of RORA was considerably reduced in HepG2 cells treated with PA. (Figure 3a). Following quantification and plotting of band intensities, the data demonstrated that RORA expression was downregulated by two times (Figure 3b).

a)

Matrix ID	Nuclear Receptor	Score	Relative Score	Start	End	Strand	Predicted sequence
MA1535.2	NR2C1	10.547004	1.0000001	-486	-481	-	AGGTCA
MA1536.2	NR2C2	9.631251	1.0	-486	-481	-	AGGTCA
MA1151.2	RORC	13.779687	0.99079406	-486	-477	-	ATCTAGGTCA
MA0071.1	RORA	13.795105	0.98425543	-486	-477	-	ATCTAGGTCA
MA1536.1	NR2C2	8.601008	0.9702801	-487	-480	-	TAGGTCAG
MA1150.2	RORB	12.825022	0.9641534	-486	-477	-	ATCTAGGTCA
MA1536.2	NR2C2	7.276239	0.95552707	-816	-811	-	GGGTTA
MA1536.2	NR2C2	7.276239	0.95552707	-338	-333	-	GGGTTA
MA1536.1	NR2C2	7.767319	0.9550551	-339	-332	-	GGGGTTAC
MA1535.1	NR2C1	9.359384	0.9550551	-487	-479	-	CTAGGTCAG

b)

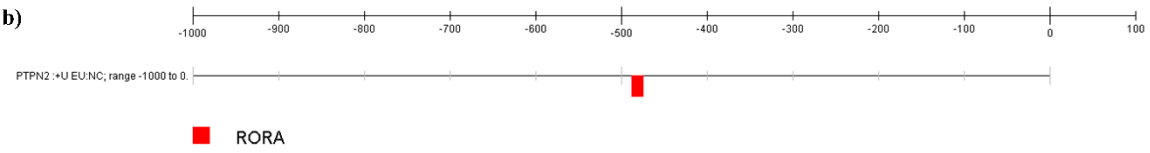

Figure 2. RORA binds to the PTPN2 promoter. (**a**) JASPER database analysis of the PTPN2 promoter showed that RORA achieved the highest score among the nuclear receptor class. (**b**) CiiiDER tool analysis showed RORA binding site in the PTPN2 promoter region.

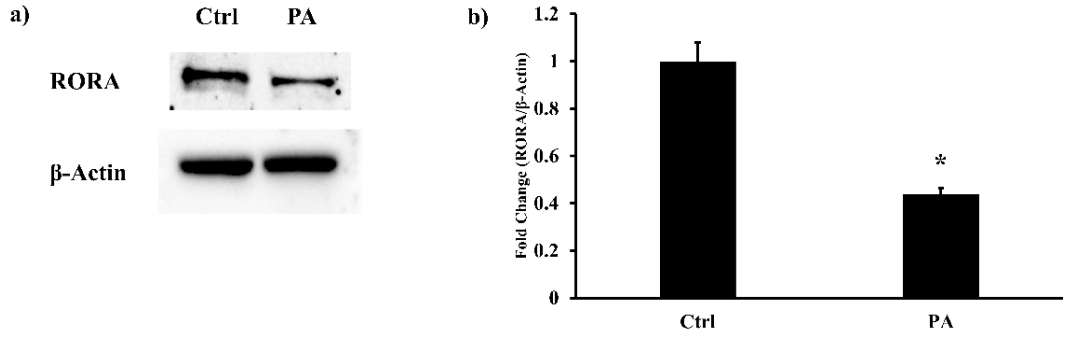

Figure 3. PA downregulates RORA in hepatic cells. (**a**) HepG2 cells were cultured and incubated with PA (300 µM) for 72 h, and total cellular protein was isolated for western blot for RORA. The PA treatment downregulated the expression of RORA. (**b**) The band intensities were quantified and represented as fold change (RORA/b-actin) (n = 3; * $p \leq 0.05$).

3.3. RORA Modulates PTPN2 Expression and Apoptosis

To cross-validate the bioinformatic results, the HepG2 cells were incubated with RORA natural agonist and synthetic antagonist, namely Cholesterol Sulphate (CS; 20 μM) and SR3335 (20 μM), respectively, for 24 h, and Western blots were performed. Previously, it was shown that 20 μM each of SR3335 [18] and CS [19] was effective in the regulation of RORA and its downstream targets in hepatic cells, and hence, this concentration was chosen for all the experiments. The findings demonstrated that the CS-treated HepG2 cells had significantly higher levels of PTPN2 and Bcl-xL expression, whereas there was a marked downregulation of the pro-apoptotic protein BAX (Figure 4a). Following quantification and plotting of band intensities, the data showed that PTPN2 and Bcl-xL expression were upregulated twofold. The expression of BAX was downregulated twofold (Figure 4b). Conversely, Western blot data indicated that SR3335 treatment of HepG2 cells markedly reduced the expression of PTPN2 and the anti-apoptotic protein Bcl-xL. In contrast, BAX, the pro-apoptotic protein, was markedly elevated. (Figure 4c). Following quantification and plotting of band intensities, the data showed that PTPN2 and Bcl-xL expression were downregulated twofold. The expression of BAX was upregulated twofold (Figure 4d).

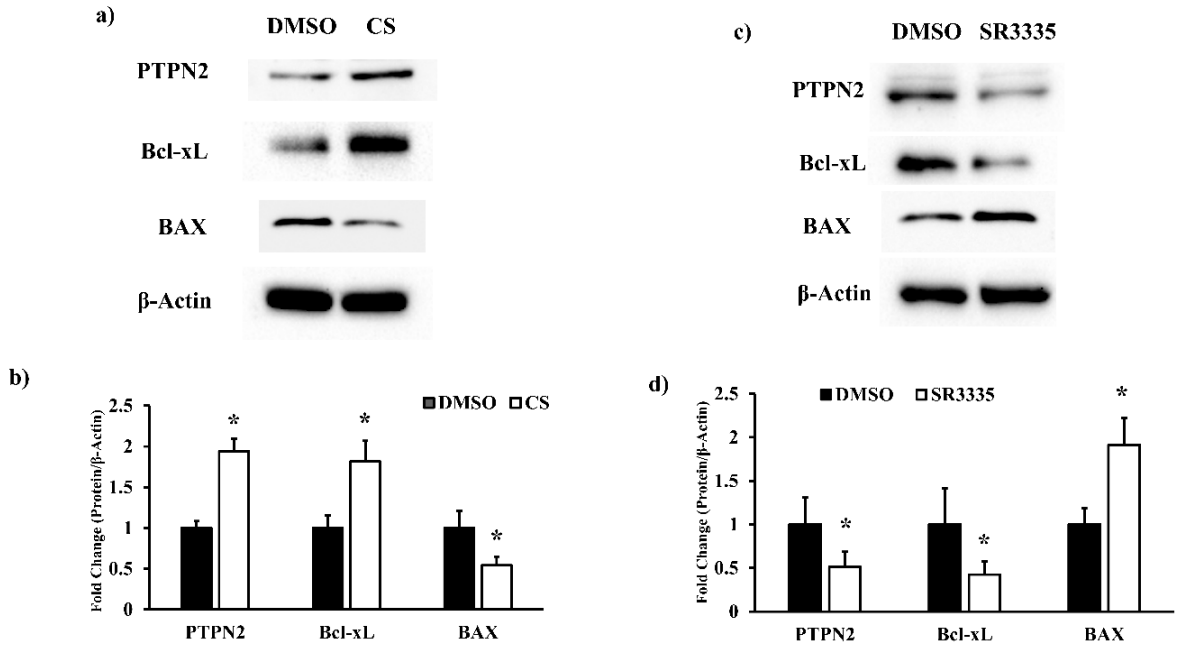

Figure 4. RORA regulates PTPN2 and apoptosis. (**a**) HepG2 cells were cultured and incubated with RORA natural agonist, Cholesterol sulphate (CS; 20 μM), for 24 h, and Western blots were performed. CS triggered the PTPN2 expression and reduced apoptosis. (**b**) The band intensities were quantified and represented as fold change (protein/b-actin) (n = 3; * $p \leq 0.05$). (**c**) HepG2 cells were cultured and incubated with RORA synthetic antagonist SR3335 (20 μM) for 24 h. Western blot results showed that SR3335 suppressed PTPN2 expression and increased apoptosis. (**d**) The band intensities were quantified and represented as fold change (protein/b-actin) (n = 3; * $p \leq 0.05$).

3.4. PTPN2 Downregulation Triggers Apoptosis

HepG2 cells were transfected with siPTPN2 and incubated for 72 h. Western blot results showed that HepG2 transfected with siPTPN2 significantly decreased the expression of PTPN2 and anti-apoptotic protein Bcl-xL, whereas BAX, a pro-apoptotic protein, was elevated (Figure 5a). Following quantification and plotting of band intensities, the data showed that PTPN2 and Bcl-xL expression were downregulated twofold. The expression of BAX was upregulated by 1.5-fold (Figure 5b).

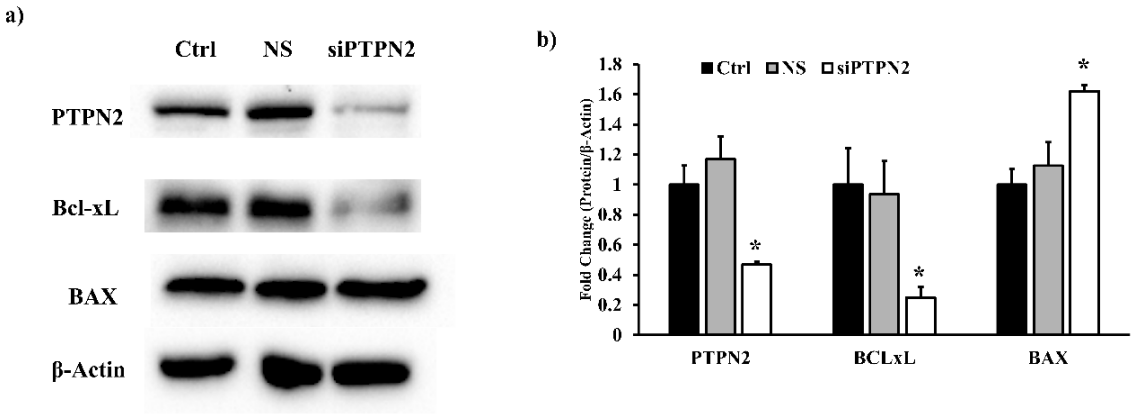

Figure 5. PTPN2 downregulation enhances apoptosis. (**a**) HepG2 cells were cultured and were transfected with siPTPN2 for 72 h. Total cellular protein was isolated, and Western blot analysis was performed. Downregulation of PTPN2 increased the apoptosis. (**b**) The band intensities were quantified using ImageJ software and represented as fold change (protein/ b-actin) (n = 3; * $p \leq 0.05$).

3.5. CDAA Diet Modulates RORA-PTPN2 and Apoptotic Proteins Expression in Mice Liver

Finally, the expression of RORA, PTPN2, and apoptotic markers was checked in liver homogenates of mice fed with all three diets, namely the standard diet, CSAA, or CDAA diet, for 54 weeks. Briefly, from the different groups of mice, the total cellular protein was isolated, and the expression of RORA, PTPN2, Bcl-xL, and BAX was determined using Western blotting (Figure 6a), and their band intensities were plotted (Figure 6b). The findings demonstrated that the mice group provided with the CDAA diet for 54 weeks had significantly higher expression of BAX and significantly lower expression of RORA, PTPN2, and Bcl-xL (Figure 6a,b).

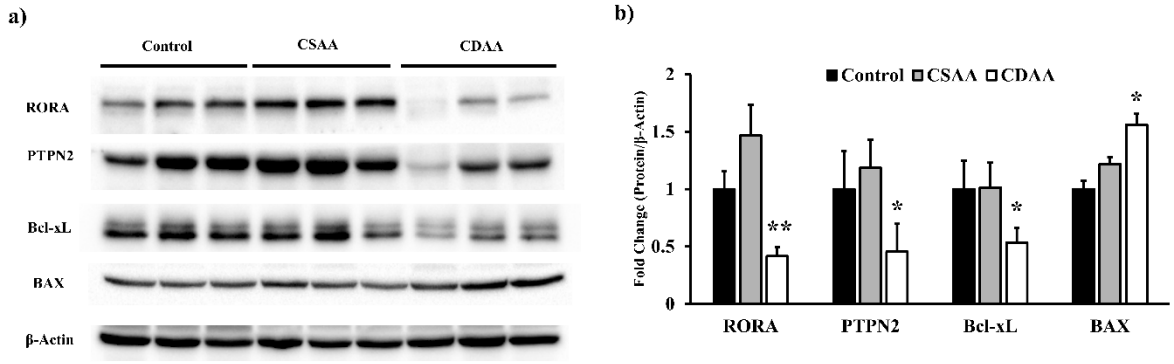

Figure 6. CDAA diet downregulates RORA-PTPN2 and increases apoptosis-related proteins in the liver. (**a**) C57BL/6 mice were fed with the standard diet, CSAA or CDAA diet, for 54 weeks. Total protein was isolated from the liver, and RORA, PTPN2, and apoptosis-related protein expression were determined. Reduced RORA and PTPN2 expression with increased apoptosis was observed in CDAA diet-fed mice. (**b**) The band intensities were quantified using ImageJ software and represented as fold change (protein/b-actin) (n = 6; * $p \leq 0.05$, ** $p \leq 0.01$).

4. Discussion

MASLD, previously called Non-alcoholic fatty liver disease (NAFLD) [20], is a syndrome caused by an impairment in the metabolism of fatty acids due to the accumulation of lipids. The histological spectrum of MASLD includes a range of conditions from simple steatosis into borderline non-alcoholic steatohepatitis (MASH)—steatosis plus weak inflammation, which can develop further into Metabolic dysfunction-associated steatohepatitis (MASH)—steatosis and strong necroinflammation, to MASH fibrosis, MASH cirrhosis, and MASH-associated HCC as an end-stage disease [3]. MASLD is strongly associated with features of metabolic syndrome, including obesity, insulin resistance (IR), type 2 diabetes mellitus (T2DM), and dyslipidemia [21].

PA is the most typical FFA reported to induce lipotoxicity during MASLD development [22]. This lipotoxicity may further trigger natural cell death in hepatic cells, which can be defined as lipo-apoptosis. We previously reported that FFAs triggered apoptosis by inducing miR-181a-5p to suppress anti-apoptotic protein

XIAP and BCL2 [23]. Similarly, incubation of PA with HepG2 cells resulted in decreased Bcl-xL expression and increased BAX expression, confirming the previous findings [8].

PTPN2, a tyrosine phosphatase, was reported to modulate multiple cellular functions by dephosphorylation of various receptor and non-receptor protein tyrosine [14]. A recent report suggested that PTPN2 modulates apoptosis via STAT3 during HCC [24]. Also, during obesity-associated MASH, PTPN2 deficiency in C57BL/6 mice fed a high-fat diet showed an increased STAT-1 signalling in hepatic cells to promote inflammation, MASH, and fibrosis, and drives hepatic cells toward HCC [15]. Similarly, a decreased expression of PTPN2 was observed in HepG2 cells treated with PA, confirming the earlier study [15]. Moreover, PTPN2 inhibition in pancreatic β-cells exhibited an aggravated cytokine-induced apoptosis via STAT1 [25]. These studies revealed a possible mechanism of regulation of apoptosis by PTPN2 via STAT proteins.

To elucidate the controlling factors of PTPN2 expression, the promoter analysis was performed. The nuclear receptors were focused mainly because they are crucial targets for treating MASLD. Nuclear receptors are a family of ligand-regulated transcription factors activated by steroid hormones and other lipid-soluble signals [26]. The analysis revealed that NR2C1, NR2C2, RORA, RORB, and RORC receptors were major binding factors. NR2C1 and NR2C2 are also commonly called Testicular nuclear receptors 2 and 4 (TR2, TR4), respectively [27]. The role of these receptors in MASLD is not known. Retinoic acid receptor-related Orphan Receptor (ROR) family receptors, namely RORA, RORB, and RORC, are reported to be involved in various functions by influencing genes involved in lipid metabolism, bile acid metabolism, glucose metabolism, detoxification, and circadian rhythm in the liver. Among them, RORA activation can attenuate MASLD [10]. Also, activation of RORA is reported to suppress apoptosis and inflammation and thereby alleviate MASLD [12]. Hence, RORA was selected for further studies.

In the promoter region of PTPN2, a consensus half-site of AGGTCA of the RORE sequence was observed. It is reported that, generally, RORA, with its zinc fingers in the DNA binding domain (DBD), interacts with RORE [28]. HepG2 cells incubated with RORA natural agonist, Cholesterol Sulphate (CS), confirmed the upregulation of PTPN2 expression and decreased apoptosis. Conversely, HepG2 cells treated with RORA synthetic antagonist, SR3335, showed a decreased expression of PTPN2 and increased apoptosis.

To further study the involvement of PTPN2 in the coordination of apoptosis, HepG2 cells were transfected with siPTPN2. As expected, pro-apoptotic marker expression was increased, while anti-apoptotic marker expression was decreased. These findings confirmed that PTPN2 is involved in the modulation of apoptosis. Finally, the CDAA-diet induced MASLD model system was used to validate these in vitro findings. A decreased expression of RORA, PTPN2, and increased apoptosis were observed in CDAA-diet fed mice.

Overall, the findings from this study showed that under normal conditions, RORA promotes the expression of PTPN2, which may further keep apoptosis at bay. However, under MASLD conditions, a suppressed expression of the RORA-PTPN2 axis facilitated apoptosis in hepatic cells (Figure 7). In summary, the free fatty acid, PA induces apoptosis by inhibiting RORA-mediated PTPN2 expression in hepatic cells. These findings of the underlying mechanism of MASLD help to design a potent agonist of RORA, which can have significant therapeutic value for the treatment of MASLD.

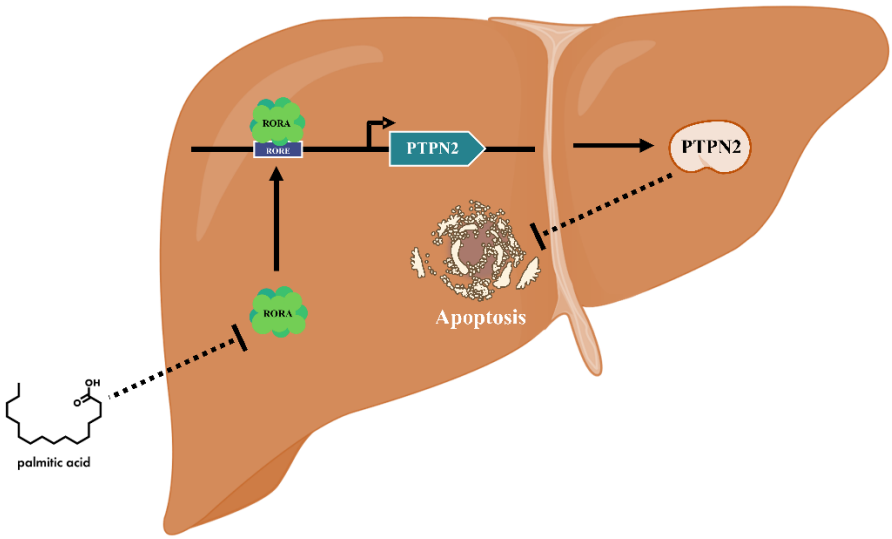

Figure 7. Schematic representation of the mechanism of palmitic acid-mediated lipo-apoptosis in hepatic cells.

Author Contributions

The original manuscript was written by T.N.S., who also conducted the majority of the experiments and standardized research procedures. M.I. carried out a few of the transfection and cell culture tests. A.K.Y. established the MASLD mice model system. S.S. and G.S. helped with a couple of Western blot and cell culture tests. A.K.S. and T.S. conducted certain experiments and aided with data analysis. The study was conceptualized and planned by S.K.V., who also edited and reviewed the article. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Prior approval for all the in vivo experiments was acquired from the Animal Ethics Committee of South Asian University, New Delhi, India.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data will be provided upon request.

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Conflicts of Interest

The authors declare no conflict of interest. Given the role as Editorial Board Member, Senthil Kumar Venugopal had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal.

Use of AI and AI-Assisted Technologies

These were not used by the authors submitting this paper.

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