Sestrin Proteins Protect Against Lipotoxicity-Induced Oxidative Stress in the Liver via Suppression of C-Jun N-Terminal Kinases

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SUMMARY
This work has identified a key role of Sesn1/2/3 proteins in the regulation of lipotoxicity-induced oxidative stress in the liver. Mechanistically, Sesn1/2/3 interact and inhibit JNK. Both in vitro and in vivo data demonstrate a strong liver-protective function of sestrins against lipotoxicity.

BACKGROUND & AIMS: Sestrin 1/2/3 (Sesn1/2/3) belong to a small family of proteins that have been implicated in the regulation of metabolic homeostasis and oxidative stress. However, the underlying mechanisms remain incompletely understood. The aim of this work was to illustrate the collective function of Sesn1/2/3 in the protection against hepatic lipotoxicity.

METHODS: We used Sesn1/2/3 triple knockout (TKO) mouse and cell models to characterize oxidative stress and signal transduction under lipotoxic conditions. Biochemical, histologic, and physiological approaches were applied to illustrate the related processes.

RESULTS: After feeding with a Western diet for 8 weeks, TKO mice developed remarkable metabolic associated fatty liver disease that was manifested by exacerbated hepatic steatosis, inflammation, and fibrosis compared with wild-type counterparts. Moreover, TKO mice exhibited higher levels of hepatic lipotoxicity and oxidative stress. Our biochemical data revealed a critical signaling node from sestrins to c-Jun N-terminal kinases (JNKs) in that sestrins interact with JNKs and mitogen-activated protein kinase kinase 7 and suppress the JNK phosphorylation and activity. In doing so, sestrins markedly reduced palmitate-induced lipotoxicity and oxidative stress in both mouse and human hepatocytes.

CONCLUSIONS: The data from this study suggest that Sesn1/2/3 play an important role in the protection against lipotoxicity-associated oxidative stress and related pathology in the liver. (Cell Mol Gastroenterol Hepatol 2021;12:921–942; https://doi.org/10.1016/j.jcmgh.2021.04.015)

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Sestrin proteins (Sesn1/2/3) play an important role in the regulation of oxidative stress, endoplasmic reticulum (ER) stress, and metabolic homeostasis.1–16
Protein structural analysis of human SESN2 reveals an alkylhydroperoxidase activity against hydrophobic cumene hydroperoxide but not hydrophilic hydrogen peroxide (H2O2). In addition to the intrinsic catalytic activity, sestrins also function through protein-protein interactions. Sestrins can activate AMP-activated protein kinase (AMPK) through an interaction with its alpha subunits. Sestrins also activate mechanistic target of rapamycin complex 2 (mTORC2) through a direct interaction with rapamycin-insensitive companion of mTOR and GATOR2, whereas they inhibit mTORC1 by regulating GATOR2.

Sestrin genes are regulated differently by cellular stress. The Sesn1 gene is activated by transformation related protein 53 in response to genotoxic stress (ultraviolet, γ-irradiation, and cytotoxic drugs). The Sesn2 gene is induced by multiple stress stimuli including genotoxic, high-fat diet, oxidative stress, ER stress, hypoxia, and others. The Sesn3 gene is up-regulated by the forkhead box O (FoxO)-mediated stress pathways but down-regulated by diet or aging. Genetic deletion of all 3 sestrin genes rather than single or double leads to high mortality rates postnatally, suggesting a significant functional redundancy. Drosophila has a single Sesn gene (dSesn). Chronic TOR activation induces dSesn gene expression through oxidative stress-triggered activation of c-Jun amino-terminal kinase (JNK) and FoxO. Loss of dSesn leads to triglyceride accumulation, mitochondrial defects, muscle degeneration, and cardiac malfunction. Sesn1 single or Sesn1/2/3 triple knockout (TKO) mice have impaired exercise capacity. Sesn2 deficiency exacerbates high-fat diet–induced insulin resistance, glucose intolerance, and hepatic steatosis in mice. Hepatic Sesn3 deficiency in mice leads to insulin resistance and glucose intolerance. Sesn3 systemic knockout (KO) mice develop severe nonalcoholic steatohepatitis (NASH) on a Western diet (WD).

In addition to the oxidoreductase activity, sestrins also protect cells against oxidative stress through positive regulation of AMPK and nuclear factor erythroid 2-related 2 (NRF2) and negative regulation of mTORC1. AMPK has been shown to mediate the sestrin antioxidative stress induced by doxorubicin in cardiomyocytes or lipopolysaccharide in mouse heart or endothelial cells. NRF2 is a master regulator of antioxidant response. After feeding with a high-carbohydrate fat-free diet, Sesn1/2 recruit the p62-mediated autophagy degradation machinery to degrade the NRF2 inhibitor, Kelch-like ECH-associated protein 1 (Keap1).

However, it remains unclear how sestrins are involved in lipotoxicity and what the underlying mechanisms are. This study aimed to address these questions.

**Results**

**Hepatic Sesn1/2/3 Deficiency Exacerbates Diet-Induced Fatty Liver Disease**

To assess the role of sestrins in human metabolic associated fatty liver disease, we queried SESN1/2/3 gene expression in the public datasets from the Gene Expression Omnibus database, and we found that hepatic SESN1 and SESN3 gene expression was trending down in hepatic steatosis patients, whereas liver SESN2 gene expression was trending down in human NASH patients (Figure 1A–C). Next, we examined the dietary effect on the expression of Sesn1/2/3 genes in the liver of wild-type (WT) mice treated with a control diet or WD for 8 weeks. Hepatic Sesn1/2/3 protein levels were decreased by 30%, 83%, and 66%, respectively, in the WD livers compared with that in the control diet livers (Figure 1D–G). To further investigate hepatic functions of the Sesn1/2/3 genes, we generated hepatic Sesn1/2/3 TKO mice by crossing Sesn1 and Sesn3 floxed mice with Sesn2 and Alb-Cre mice. Immunoblot analysis of Sesn1/2/3 proteins in the liver and white adipose tissue confirmed that both Sesn1 and Sesn3 genes were specifically deleted in the liver on the background of the Sesn2 gene global KO (Figure 2A and B). Next, we fed the animals with either the control diet or WD for 8 weeks. During the dietary treatment, WT, Sesn2 knockout (Sesn2KO), and TKO mice had no significant difference in body weight on the same diet (Figure 2D–G). However, liver weight was significantly increased by 22% in the TKO mice as compared with that in the WT mice on WD, whereas white adipose tissue weight and liver or white adipose tissue to body weight ratios were not significantly different on either the control diet or WD (Figure 2D–G). WD induced a typical fatty liver phenotype manifested by a pale liver appearance and a significant increase in hepatic triglyceride and cholesterol levels in WT, Sesn2KO, and TKO mice as compared with the respective genotype on the control diet, with the lipid levels being highest in the TKO livers (Figure 2H–J, Figure 3A and B). Serum alanine aminotransferase levels were increased by 84% and 57% in the TKO mice as compared with those in the WT mice under the control diet and WD conditions, respectively (Figure 3C), suggesting that there was elevated liver injury in TKO mice. We performed immunohistochemical analysis of apoptosis in liver sections, and our data showed that TKO livers had increased cell death on the
Control diet and even more so on WD compared with that in WT livers (Figure 3D and E). In addition, expression of lipogenic genes including sterol regulatory element-binding protein 1c (Srebp1c) and fatty acid synthase (Fasn) was significantly increased in the livers of TKO mice as compared with WT mouse livers on the control diet (Figure 3F). Srebp1a, diacylglycerol O-acyltransferase 2 (Dgat2), and stearoyl-CoA desaturase 1 (Scd1) genes were significantly up-regulated in the livers of TKO mice compared with the WT livers on WD (Figure 3G).
Next, we examined hepatic inflammation by immuno-staining neutrophil and macrophage markers, myeloperoxidase (MPO) and F4/80, respectively. MPO⁺ and F4/80⁺ cell numbers were increased by 2.6- and 2.9-fold, respectively, in the TKO livers compared with those in the WT livers (Figure 4A and B). Tumor necrosis factor (Tnf), a pro-inflammatory gene, was up-regulated, whereas interleukin 10 (Il10), an anti-inflammatory gene, was significantly down-regulated in the TKO livers compared with those in the WT livers on the control diet (Figure 4C). BCL2

**Figure 2.** (See previous page). Genotyping and general characterization of Sesn1/2/3 TKO mice. (A and B) Western blot analysis of Sesn1, Sesn2, and Sesn3 proteins in liver and white adipose tissue (WAT) of WT, Sesn2KO, and TKO mice. (C–G) Measurements of body weight, liver weight, liver to body weight ratio, and WAT to body weight ratio, respectively. (H) Liver images collected at end of the experiment. (I) H&E staining of liver sections. (J) Quantification of lipid droplet areas in liver sections of control diet or WD treated mice. Data are expressed as mean ± SD (n = 5–8). *P < .05, **P < .01, ***P < .001 for WD vs control diet for the respective genotypes.

**Figure 3.** Sesn1/2/3 TKO mice are susceptible to WD-induced hepatic steatosis and liver injury. (A) Hepatic triglyceride (TG) measurements in WT, Sesn2KO, and TKO mice fed with control diet or WD. (B) Hepatic total cholesterol (TC) measurements. (C) Serum alanine aminotransferase (ALT) levels. (D and E) Hepatic apoptosis analysis and quantification in liver sections using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays (original magnification, ×400). (F and G) Real-time PCR analysis of lipogenic genes in livers of control diet or WD treated mice. Data are expressed as mean ± SD (n = 4–8). *P < .05, **P < .01, or ****P < .0001 for the respective genotype on WD vs control diet.
associated X apoptosis regulator (Bax) and DNA damage inducible transcript 3 (Ddit3, also called Chop), 2 pro-apoptosis genes, were significantly up-regulated in the livers of TKO mice compared with the WT livers on the control diet (Figure 4C). Similarly, the Tnf, Il1b, and Il6 genes were up-regulated, whereas the Il10 gene was down-regulated in the TKO livers compared with the WT livers on WD (Figure 4D). Hepatic TNF-α and IL6 protein levels were also elevated in the TKO mice compared with WT mice, whereas IL1β and IL10 levels were not significantly changed (Figure 4E–H). We also examined hepatic fibrosis by using multiple approaches in the WD treated mice. First, liver sections were stained with a Sirius Red dye, and the data showed that hepatic fibrosis area was increased by 50% in the TKO mice compared with that in the WT mice (Figure 5A and B). Second, we performed immunofluorescent staining of 3 fibrosis-associated proteins, collagen type 1 (Col1), smooth muscle actin alpha 2 (Acta2), tissue inhibitor of metalloproteinase 1 (Timp1), and our data showed that all 3 fibrosis markers were significantly increased in the TKO livers compared with those in the WT livers (Figure 5C and D). Third, we analyzed expression of fibrosis-related genes including transforming growth factor beta 1 (Tgfb1), transforming growth factor beta
receptor 1 (Tgfbr1), platelet-derived growth factor receptor beta (Pdgfrb), Timp1, Acta2, and Col1a1 by real-time polymerase chain reaction (PCR), and our data showed that the Tgfb1, Tgfbr1, and Pdgfrb genes were significantly induced in the TKO livers compared with those in the WT livers on both the control diet and WD, whereas the Timp1, Acta2, and Col1a1 genes were up-regulated in the TKO livers compared with the WT livers only on WD (Figure 5E and F).

Sesn1/2/3 Gene Knockouts Exacerbate Diet-Induced Oxidative Stress

To investigate how Sesn1/2/3 deficiency impacts WD-induced hepatic oxidative stress, we analyzed several markers of oxidative stress. Hepatic H2O2 levels were increased by 63% and 88% in the TKO mice compared with those in the WT mice on the control diet and WD, respectively (Figure 6A). Hepatic nitric oxide levels were increased by 71% in the TKO mice compared with that in the WT mice.
under the WD condition (Figure 6B). We also measured 2 lipid peroxidation markers, 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), by immunostaining and biochemical assays, respectively. Our data showed that 4-HNE and MDA levels were increased by 3-fold and 2-fold, respectively, in the TKO livers compared with the WT livers under the WD condition (Figure 6C and D). We also analyzed antioxidative responses to WD by measuring total glutathione (GSH) and superoxide dismutase (SOD) activity. Our data showed that GSH levels and SOD activities were decreased by 17% and 35%, respectively, in the TKO livers compared with the WT livers under the WD condition (Figure 6E and F). In addition, we analyzed expression of several oxidative stress related genes in the livers of WT and KO mice fed with the control diet or WD. On the control diet, glutathione synthetase (Gss) gene was significantly down-regulated, whereas glutathione peroxidase 3 (Gpx3) was significantly up-regulated in the TKO livers compared with the WT livers (Figure 6G). On WD, NADPH oxidase (Nox1), a pro-oxidative stress gene, was increased by 76%, whereas antioxidative stress genes such as Gss, Gpx3, Sod1, Sod3, and catalase (Cat) were significantly decreased in the TKO livers compared with the WT livers (Figure 6H).

To further examine whether the lipotoxicity-induced oxidative stress in the TKO liver primarily happens in hepatocytes, we treated primary hepatocytes from WT and TKO mice with either fatty acid–free bovine serum albumin (BSA) or BSA conjugated palmitic acids (PA) (200 μmol/L) for 18 hours and analyzed oxidative stress markers. Neutral lipid levels measured by BODIPY were increased by 74% in the TKO hepatocytes compared with the WT hepatocytes after the PA treatment (Figure 7A and B). CellROX, dihydroethidium (DHE), and 4-HNE positive signals were increased by 33%, 182%, and 31%, respectively, in the TKO hepatocytes compared with the WT hepatocytes treated with PA (Figure 7A and C–E). Immunoblot analysis also showed that 4-HNE modified proteins were increased by 66% in the PA-treated TKO hepatocytes compared with those in the WT hepatocytes (Figure 7F and G). Sod2 was decreased in the TKO hepatocytes relative to the WT hepatocytes without the PA treatment, and PA further reduced Sod2 in both WT and TKO hepatocytes (Figure 7F and H).

**Sesn1/2/3 Inhibit Lipotoxicity-Triggered Oxidative Stress via Suppression of JNK**

To investigate the mechanism underlying the oxidative stress in the TKO liver, we analyzed a stress-related kinase, JNK. Under the normal control diet condition, there was no significant difference in phosphorylated Jnk (p-Jnk Thr183/Tyr185) in the livers of WT, Sesn2KO, and TKO mice (Figure 8A and B). Next, we examined the effect of WD on Jnk phosphorylation. As expected, WD increased p-Jnk levels by 75% in the livers of WT mice as compared with the control diet (Figure 8C and D). In contrast, phosphorylated levels of mitogen-activated protein kinase kinase 7 (Mkk7, a kinase for Jnk), Jnk, c-Jun (a substrate of Jnk), nuclear factor kappa B (NF-κB), and p38 were increased by 2-, 8-, 15-, 2.3-, and 2.2-fold, respectively, in the TKO livers compared with those in the WT livers under the WD condition (Figure 9A–F). We also performed immunostaining of p-Jnk in liver sections, and our data showed that hepatic p-Jnk levels were increased by 131% in the TKO mice compared with that in the WT mice (Figure 9G and H).

To corroborate the interaction between lipotoxicity and Sesn1/2/3 genes, first, we isolated primary hepatocytes from WT and TKO mice and treated the cells with 200 μmol/L PA for 18 hours. After the PA treatment, p-Mkk7, p-Jnk, p-c-Jun, and p-p38 levels were increased by 239%, 45%, 130%, and 156%, respectively, in the TKO hepatocytes compared with those in the WT hepatocytes (Figure 9I–M). Second, we generated SESN1/2/3-deficient HepG2 cells using the CRISPR/Cas9 technology. Gene knockdown (KD) was confirmed by immunoblotting (Figure 10A). We co-treated WT and SESN1/2/3 KD cells with 200 μmol/L PA and a JNK inhibitor SP600125 or control vehicle for 18 hours. Phosphorylated c-JUN positivity was increased by 85% in the KD cells compared with WT cells, and this difference was largely abolished by SP600125 (Figure 10B and C). The alteration of p-c-JUN by PA and SP600125 was also confirmed by immunoblot analysis (Figure 10D and E). Lipid peroxidation and intracellular H2O2 levels were significantly elevated in the SESN1/2/3 KD cells without the PA treatment and more dramatically increased with the PA treatment. Both oxidative stress indicators, 4-HNE and H2O2, were significantly decreased in the presence of the JNK inhibitor (Figure 10F–H).

To examine whether sestrins directly regulate Jnk, we performed co-immunoprecipitation analysis of Sesn1/2/3 and Jnk2 in Huh7 cells after co-transfection of their plasmid DNAs. Our data showed an interaction between Sesn1/2/3 and Jnk2 (Figure 11A). Moreover, we performed pull-down assays from Huh7 cells using Flag-tagged Sesn1/2/3 after they were treated with 200 μmol/L PA for 18 hours. Sesn1/2/3-Flag pulled down several kinases including Jnk, Mkk7, p38 MAPK, and AKT (a positive control) but not ERK (Figure 11B). We also observed that overexpression of Sesn1/2/3 decreased p-Jnk (Thr183/Tyr185), p-p38 (Thr180/Tyr182), and p-ERK (Thr202/Tyr204) levels (Figure 11B). To further explore how sestrins regulate JNK, we co-transfected Jnk2 and Sesn3 in Huh7 cells and analyzed JNK and MKK7 interactions. We observed that Sesn3 overexpression dramatically reduced the interaction between Jnk2 and endogenous MKK7 (Figure 11C). To verify whether the interaction between Sesn3 and Jnk2 is required for the disruption of the JNK-MKK7 interaction, we generated N- and C-terminal truncated Sesn3 constructs (amino acids 1-240 and 241-492, respectively) and found that C-terminal but not N-terminal Sesn3 interacted with Jnk2. As predicted, only C-terminal Sesn3 attenuated the interaction between Jnk2 and MKK7 (Figure 11D). Moreover, C-terminal Sesn3 like full length suppressed phosphorylation of M KK7 and Jnk after their overexpression in the Huh7 cells (Figure 11E). To further validate the protein-protein interactions in vivo, we performed 2 independent pull-down assays from mouse liver lysates using HA-tagged Sesn3 transgenic mice or Sesn1/2/3 antibodies. Sesn3-HA pulled

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**Table:**

| Condition | JNK Phosphorylation |
|-----------|---------------------|
| WT        | None                |
| TKO       | Increased by 131%    |
| SESN1/2/3 KD | Decreased by 85%    |

**Figure:**

- Figure 6B: WD-induced oxidative stress in TKO liver.
- Figure 7A and B: CellROX and DHE signals in WT and TKO hepatocytes.
- Figure 8A and B: Jnk phosphorylation in WT, Sesn2KO, and TKO mice.
- Figure 9A–F: Immunostaining of p-Jnk in liver sections.
- Figure 10A: Gene knockdown confirmation.
- Figure 11A: Co-immunoprecipitation analysis.
- Figure 11B: Overexpression of Sesn1/2/3 decreased p-Jnk levels.
- Figure 11C: Overexpression of Sesn3 reduced Jnk2-MKK7 interaction.
down Jnk, p-Jnk, Mkk7, and p38 Mapk but not Erk (Figure 12A). Similarly, Sesn1/2/3 antibodies also pulled down those kinases except Erk (Figure 12B). Taken together, these data suggest that sestrins interact with Mkk7, Jnk, and p38 Mapk in both hepatocytes and livers.

To further verify that sestrins suppress JNK and oxidative stress, we also used a gain-of-function approach by overexpressing individual sestrin genes in HepG2 cells and treating with 200 µmol/L PA for 18 hours. Our data showed that Sesn1/2/3 overexpression significantly decreased JNK and c-JUN phosphorylation and 4-HNE levels (Figure 13A–E). Because sestrins have also been implicated in the regulation of mTOR and ER stress, we analyzed the effect of Sesn1/2/3 deficiency and PA on these signaling pathways in control and SESN1/2/3 KD HepG2 cells. Our data showed that PA exacerbated mTORC1 and protein kinase R-like endoplasmic reticulum kinase (PERK) activities as shown by elevated phosphorylation of S6 and eIF2α.

**Figure 6. Hepatic oxidative stress is highly elevated in the Sesn1/2/3 TKO liver.** (A and B) H2O2 and nitric oxide (NO) were measured in liver extracts of control diet or WD fed mice, respectively. (C) Immunofluorescence and quantification analysis of 4-HNE in liver sections of WD treated mice (original magnification, ×100). (D) MDA was measured in liver extracts of control diet or WD fed mice. (E and F) Total GSH and SOD activities were measured in liver extracts of control diet or WD treated mice, respectively. (G and H) Real-time PCR analysis of redox genes in livers of control diet or WD treated mice. Data are expressed as mean ± SD (n = 4–8). *P < .05 and **P < .01 for the respective genotype on WD vs control diet.
respectively (Figure 14A and B). To further examine a potential crosstalk between sestrins and mTORC1, PERK, and oxidative stress in the regulation of JNK, we treated control and SESN1/2/3 KD HepG2 cells with rapamycin (an mTORC1 inhibitor), GSK2656157 (a PERK inhibitor), and N-acetyl cysteine (an antioxidant) together with 200 μmol/L

Figure 7. Sesn1/2/3 deficiency in hepatocytes exacerbates lipotoxicity-induced oxidative stress. (A–E) Immunofluorescence imaging and quantification analysis of lipid droplets by BODIPY dye and oxidative stress by CellROX, DHE, and 4-HNE staining in WT and TKO mouse primary hepatocytes treated with control BSA or 200 μmol/L PA conjugated in BSA for 18 hours, respectively (original magnification, ×630). (F–H) Immunoblotting and quantification analysis of 4-HNE and Sod2 in BSA or PA treated WT and TKO mouse primary hepatocytes. Data are expressed as mean ± SD (n = 3–4). *P < .05, **P < .01, or ***P < .001 for TKO vs WT hepatocytes under the same treatments.
PA. The data showed that inhibition of mTORC1 or PERK or attenuation of oxidative stress reduced the lipotoxicity-induced JNK activation (Figure 15A–I). These data suggest that under the lipotoxic conditions, JNK is subjected to regulation by multiple factors including sestrins, mTORC1, ER stress, and oxidative stress.

**Discussion**

It has been widely reported that saturated fatty acids such as palmitates can induce oxidative stress and even cell death. In this work, we demonstrate the protective effect of Sesn1/2/3 against hepatic lipotoxicity. Our results are consistent with several previous studies on the hepatoprotective role of sestrins through various mechanisms. Sesn2/- mice are susceptible to high-fat-induced hepatic steatosis and ER stress due to decreased AMPK and increased mTORC1 activities. Sesn2 also protects high-carbohydrate fat-free diet–induced liver injury through p62-dependent autophagic degradation of Keap1 and activation of NRF2.1 We have recently reported a key role of Sesn3 in the suppression of the TGFβ-SMAD3 pathway during NASH development. In addition, Sesn3 deficiency significantly increases carcinogen-triggered hepatocellular carcinoma development through activation of the hedgehog signaling pathway. Our data from the current study have shown that Sesn1/2/3 have redundant functions in the liver because TKO mice manifest worse fatty liver phenotype than Sesn2KO mice, especially in hepatic inflammation, fibrosis, and oxidative stress.

In this work, we have explored the mechanism for the Sesn1/2/3 functions in the control of hepatic lipotoxicity and have identified JNK as a key regulatory point. Our biochemical characterization has revealed a direct protein-protein interaction between Sesn1/2/3 and JNK2. Functional analysis suggests that the interactions inhibit the JNK phosphorylation and activity. Because JNK kinases both respond to and enhance oxidative stress, attenuation of the JNK activity by sestrins can be very helpful to
Figure 9. Jnk activity is elevated in the Sesn1/2/3-deficient liver on WD. (A–F) Immunoblot and quantification analysis of total and phosphorylated Mkk7, Jnk, c-Jun, NF-kB, and p38 in livers of WD treated mice. (G and H) Immunofluorescence and quantification analysis of phosphorylated Jnk in liver sections of WD treated mice (original magnification, ×200). (I–M) Immunoblot and quantification analysis of total and phosphorylated Mkk7, Jnk, c-Jun, and p38 in WT and TKO mouse primary hepatocytes treated with BSA or 200 μmol/L PA for 18 hours. Data are expressed as mean ± SD (n = 3–4). *P < .05, **P < .01, or ***P < .001 vs WT.
control oxidative stress within the cell tolerance range. Interestingly, sestrins have been shown to activate ERK, JNK, and p38 MAPK in the same protein complex in senescent T lymphocytes. Our data suggest that sestrins interact with JNK and p38 MAPK but not ERK1/2 in hepatocytes. The differential regulation of JNK among...
different cell types requires further comparative studies. In addition, our data also suggest that sestrins interact with MKK7, which is an upstream kinase for JNK. These data implicate that Sesn1/2/3 could suppress the JNK signaling pathway through inhibition of JNK phosphorylation by MKK7. At this time, the detailed mechanism remains to be determined. We surmise that binding of Sesn1/2/3 to JNK could directly interfere with the JNK Thr183 phosphorylation by MKK7. In addition, Sesn1/2/3 may indirectly regulate the JNK activity phosphorylation by MKK7. At this time, the detailed mechanism remains to be determined. We surmise that binding of Sesn1/2/3 to JNK could directly interfere with the JNK Thr183 phosphorylation by MKK7. In addition, Sesn1/2/3 may indirectly regulate the JNK activity

Figure 10. (See previous page). SESN1/2/3 gene KD in human hepatocytes increases lipotoxicity and JNK activity. (A) Confirmation of SESN1/2/3 triple KDs in HepG2 cells by immunoblotting. (B–E) Analysis of phosphorylated c-JUN by immunocytochemistry (original magnification, ×400) and immunoblotting in HepG2 cells treated with control DMSO or a JNK inhibitor SP600125 (10 μmol/L) for 6 hours, followed by incubation with BSA or 200 μmol/L PA for 18 hours. (F–H) Immunocytochemical and biochemical analysis of 4-HNE and H2O2 in control or SESN1/2/3 KD HepG2 cells after treatment with DMSO or SP600125 for 6 hours, followed by incubation with BSA or 200 μmol/L PA for 18 hours. Data are expressed as mean ± SD (n = 3). *P < .05 and **P < .01 vs green fluorescent protein (GFP).

Figure 11. Sestrins interact with Jnk in hepatocytes. (A) Co-immunoprecipitation (IP) analysis of interactions between Sesn1/2/3 and Jnk2 in Huh7 cells after co-transfection of the indicated plasmids. IP reactions were performed with anti-HA monoclonal antibody beads. (B) Pull-down analysis of endogenous kinases by Flag-tagged Sesn1, Sesn2, and Sesn3 in Huh7 cells after treatment with 200 μmol/L PA for 24 hours. (C) Co-IP analysis of interactions between Jnk2 and Sesn3 and MKK7 after transfection of Flag-Jnk2 and HA-Sesn3 in Huh7 cells for 48 hours. (D) Pull-down analysis of Jnk2 and MKK7 after transfection of Flag-Jnk2 and N-terminal or C-terminal truncated Sesn3 (Sesn3-N and Sesn3-C, respectively) plasmids in Huh7 cells for 48 hours. (E) Immunoblot analysis of MKK7 and JNK phosphorylation levels after transfection of either full-length (FL) or truncated Sesn3 plasmids in Huh7 cells for 48 hours. Data are expressed as mean ± SD (n = 3). *P < .05 and **P < .01 vs green fluorescent protein (GFP).
through the mTOR signaling, ER stress, or oxidative stress because our data showed that inhibition of mTORC1 or PERK or attenuation of oxidative stress reduces the JNK phosphorylation even in the Sesn1/2/3 KD cells.

In summary, our data strongly suggest a protective effect of Sesn1/2/3 against lipotoxicity and oxidative stress in the liver. Through the suppression of the JNK activity, sestrins break the vicious cycle of reactive oxygen species production and lipid peroxidation and their pathogenic sequelae. Considering this and other salutary functions of sestrins, we believe that positively modulating sestrin activity can be an attractive strategy for NASH therapeutic development.

Materials and Methods

Animal procedures

Animals. All animal care and experimental procedures performed in this work were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. Sesn1, Sesn2, and Sesn3 KO mice were generated as previously described.32 Sesn1 and Sesn3 liver-specific KOs on the background of Sesn2 systemic KO mice (TKO) were generated by crossing Sesn1/3 flanked mice with an Alb-Cre strain and Sesn2−/− mice. Sesn3 transgenic mice were generated as previously described.4 Mice were housed in an animal facility with controlled temperature (22 ± 2°C), humidity (60% ± 5%), and a regular 12:12 light/dark cycle. Animals were fed a control diet that is composed of 18 kcal% fat, 58 kcal% carbohydrate, and 24 kcal% protein or a WD (Research Diets Inc, New Brunswick, NJ) that contains 40 kcal% fat, 20 kcal% fructose, and 1% cholesterol for 8 weeks. Body weight was measured weekly. At the end of the experiments, mice were euthanized under anesthesia for blood and tissue collection.

Genotyping. Sesn1/2/3 KO mice were genotyped by PCR analysis and confirmed by immunoblotting. Genotyping PCR primers are described in Table 1. The following antibodies were used for immunoblotting: rabbit anti-Sesn1 polyclonal antibody (PA-526881; Invitrogen, Waltham, MA), rabbit anti-Sesn2 polyclonal antibody (PA-572834; Invitrogen), and rabbit anti-Sesn3 polyclonal antibody (P1PA522220; Thermo Fisher Scientific, Waltham, MA).

Biochemical Analysis

Serum alanine aminotransferase was measured using a commercial kit according to the manufacturer’s manual (Thermo Fisher Scientific). Hepatic total cholesterol and triglycerides were analyzed using commercial assay kits (Wako USA, Richmond, VA). Tissue and cellular hydrogen peroxide (H2O2) and nitric oxide were measured using commercial assay kits (Invitrogen, and BioVision, Milpitas, CA). Lipid peroxidation was determined using an assay kit.
for MDA (BioAssay Systems, Hayward, CA). GSH and SOD were analyzed using assay kits from Sigma-Aldrich (St Louis, MO). Tissue cytokines were measured using commercial enzyme-linked immunosorbent assay kits (mouse TNF-α and mouse IL6 from BD Biosciences, San Jose, CA; mouse IL1β and mouse IL10 from R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

**mRNA Analysis**

Total RNAs were isolated from cells and tissues using TRI reagent from Sigma-Aldrich. cDNA was synthesized using a reverse transcription kit (Invitrogen). Real-time PCR analysis was performed using a SYBR Green qPCR kit (Invitrogen). Primer sequences are described in Table 1.
Protein Analysis

Total protein and phosphorylated protein levels were analyzed by immunoblotting after sodium dodecyl sulfate-gel electrophoresis. The following antibodies were used for the immunoblots: anti-p-NF-κB (Ser536; Cell Signaling Technology, Danvers, MA; #3033), anti- NF-κB (Cell Signaling Technology; #8242), anti-p-JNK (Thr183/Tyr185; Cell Signaling Technology; #4668), anti-JNK (Cell Signaling Technology; #9258), and anti-p-c-Jun (Ser63; Cell Signaling Technology; #9261). Immunoprecipitation was performed in HepG2 or Huh7 cells after transfection of indicated DNA constructs. Immunoprecipitation and immunoblotting were performed using tag antibodies: mouse monoclonal anti-FLAG M2 affinity gel (Sigma-Aldrich; #A2220) and rabbit anti-HA tag antibody (Cell Signaling Technology; #3724).

Histologic Analysis

Tissue samples were fixed in neutral formalin solution (10%) and then processed for embedding and sectioning at the Histology Core of Indiana University School of Medicine. Liver sections (4-μm thickness) were stained with H&E or Sirius Red dye according to the standard protocols. Cell death in liver sections was analyzed using an ApopTag peroxidase in situ apoptosis detection kit (Sigma-Millipore, Burlington, MA) by following the kit manual. To examine hepatic oxidative stress, inflammation, and fibrotic alterations, we performed immunofluorescence analysis of fixed liver sections. After deparaffinization and rehydration, the liver sections were incubated with 2.5% normal horse serum for 1 hour at room temperature and then with the following primary antibodies: mouse anti-4-HNE monoclonal antibody (R&D Systems; MAB3249, 1:250), mouse...
| Gene   | Species | Primer sequence (forward and reverse) | Application |
|--------|---------|--------------------------------------|-------------|
| Srebp1a | Mouse   | 5'-GAA CAG ACA CTG GCC GAG AT-3'   5'-AGC TGG AGC ATG TCT TCG AT-3' | qPCR        |
| Srebp1c | Mouse   | 5'- GGA GCC ATG GAT TGC ACA TT -3'' 5'- CCT GTC TCA CCC CCA GCA TA -3' | qPCR        |
| Fasn   | Mouse   | 5'- CTG CCA CAA CTC TGA GGA CA-3'' 5'- TTC GTA CTT CCT TGG CAA AC -3' | qPCR        |
| Dgat1  | Mouse   | 5'- ACA GAG ACC ACC AGC ACA AC-3'' 5'- ATT CAT CCT GTC TCG GAC TGC -3' | qPCR        |
| Dgat2  | Mouse   | 5'- TTC CTG GCA TAA GGC CCT ATT-3'' 5'- AGT CTA TGG TGT CTC GGT TGA C-3' | qPCR        |
| Fasn   | Mouse   | 5'- CTG CCA CAA CTC TGA GGA CA-3'' 5'- TTC GTA CTT CCT TGG CAA AC -3' | qPCR        |
| Scd    | Mouse   | 5'-GCG TTC CAG AAT GAC GTG TA-3'' 5'-CCA ACC CAC GTG AGA GAA GA-3' | qPCR        |
| Gss    | Mouse   | 5'- TGT GCC CTT TTA CCC TCT TCC T -3'' 5'- TCT TTG GAG TGT GGG AAT GGA -3' | qPCR        |
| Gpx3   | Mouse   | 5'- GCT TGG TCA TCC TGG GCT TC -3' 5'- CCC ACC TGG TGG TGG AAC ATA CT -3' | qPCR        |
| Sod1   | Mouse   | 5'- TTG GCC GTA CAA TGG TGG T-3' 5'- CGC AAT CCC AAT CAC TCC AC -3' | qPCR        |
| Sod2   | Mouse   | 5'- GGT GCC GTT GAG ATT GTT CA -3' 5'- CCA AGT GCC TTA CTA AAC CAT AT -3' | qPCR        |
| Sod3   | Mouse   | 5'-CTT GTT CTA CGG CTG TCT GCT GCT ACT G-3' 5'- ATG CGT GTG GCC TAT CTT CT-3' | qPCR        |
| Cat    | Mouse   | 5'- TCA CCC AGC ATA TCA CCA GA -3' 5'- AGC TGA GCC TGA CTC TCC AG -3' | qPCR        |
| Nox1   | Mouse   | 5'- TGG AGT GGG TTG ACC-3' 5'- TGC TGG CAT GAC CAA CCT TTT-3' | qPCR        |
| Nox2   | Mouse   | 5'- TTT ACA CTG ACA TCC GCC CC-3' 5'- TGG GCC GTT CAT ACA AAG TC-3' | qPCR        |
| Mpo    | Mouse   | 5'- GCC AAG GCC TTT CAA TGT TA-3' 5'- TCA CGT CCT GAT AGG CAC AG-3' | qPCR        |
| Tnf    | Mouse   | 5'- GCC TTC CCT CCT ATC AGT TC-3' 5'- CAC TTG GTG GTT TGC TAC GA-3' | qPCR        |
| Il1b   | Mouse   | 5'- TGT GAA ATG CCA CTT TTT GA-3' 5'- GGT CAA ACG GAT TTA GGA AG-3' | qPCR        |
| Il6    | Mouse   | 5'-CAA AGC CAG AGT CCT TCA GAG-3' 5'-GAG CAT TGG AAA TTG GGG TA-3' | qPCR        |
| Il10   | Mouse   | 5'- CAG AGC CAC ATG CTC CTA GA-3' 5'-GCT TGG CAA CCC AAG TAA CC-3' | qPCR        |
| Tgfb1  | Mouse   | 5'-CGC AAC AAC GCC ATC TAT GA 5'-ACT GCT TCC CGA ATG TCT GA | qPCR        |
| Tgfr   | Mouse   | 5'-CGC AAC AAC GCC ATC TAT GA 5'-ACT GCT TCC CGA ATG TCT GA | qPCR        |
| Pdgfbr | Mouse   | 5'-CAT TGG CAA AAG CAC CAT TG 5'-GAC ATT CAC AGC GAC GTT GA | qPCR        |
| Mmp2   | Mouse   | 5'-ACT CCG GAG ATC TGC AAA CA 5'-ACT GTC CGC CAA ATA AAC CG | qPCR        |
| Timp1  | Mouse   | 5'-CAT GGA AAG CCT CTG TGG AT 5'-CTC AGA GTA GGC CAG GGA AC | qPCR        |
| Acta2  | Mouse   | 5'-AGG CAC CAC TGA ACC CTA AG 5'-GAC AGC ACA GCC TGA ATA GC | qPCR        |
| Col1a1 | Mouse   | 5'-CAC CTG GTC CAC AAG GTT TC 5'-CCC ATC ATC TCC ATT CCT GC | qPCR        |
| Col4a1 | Mouse   | 5'-TTC GCC TCC AGG AAC GAC TA 5'-AAA CGG CAC ACC TGC TAA TG | qPCR        |
anti-F4/80 monoclonal antibody (Invitrogen; MA5-16363, 1:250), rabbit anti-MPO polyclonal antibody (Invitrogen; PA5-16672, 1:250), rabbit anti-TIMP-1 polyclonal antibody (Proteintech, Rosemont, IL; #16644-1-AP, 1:200), rabbit anti-α-SMA polyclonal antibody (Abcam, Cambridge, MA; ab5694, 1:200), and rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185; Cell Signaling Technology; #4668, 1:200). Next, the following fluorophore-conjugated secondary antibodies were applied to the sections: goat anti-rabbit immunoglobulin G (H+L) Alexa Fluor 488 (Invitrogen; A11034), goat anti-mouse immunoglobulin G (H+L) Alexa Fluor 488 (Invitrogen; A11001), donkey anti-rabbit immunoglobulin G (H+L) Alexa Fluor 594 (Invitrogen; A21207), and goat anti-mouse immunoglobulin G (H+L) Alexa Fluor 594 (Invitrogen; A11032). Images for H&E or Sirius Red staining were captured using a Leica DM750 microscope (Wetzlar, Germany) equipped with a EC3 digital camera and LAS EZ software (Buffalo Grove, IL). Immunofluorescence images were obtained by a Zeiss fluorescence microscope (Oberkochen, Germany) using AxioVision Rel 4.8 software (White Plains, NY). Positive signals were quantified from the randomly selected sections at least 5 fields per sample using Image J 1.64 software (NIH, Bethesda, MD).

**Cell Culture and Generation of SESN1/2/3-Deficient Cell Line Using CRISPR-Cas9**

Human hepatoma cell line HepG2 cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific) in an incubator under conditions of 37°C and 5% CO₂. To generate SESN1/2/3 KD HepG2 cells, we designed single guide RNAs (sgRNAs) targeting human SESN1/2/3 coding sequences using the GPP sgRNA Designer on the Broad Institute website and cloned the sgRNAs into a lentiviral vector as a gift from Dr Feng Zhang (Addgene, Cambridge, MA; #52961; http://n2t.net/addgene:52961; RRID:Addgene_52961). To induce lipotoxicity, HepG2, Huh7, and mouse primary hepatocytes were treated with 200 μmol/L PA (Sigma-Aldrich) or control vehicle BSA. Cells were seeded at 2.5 × 10⁴ cells/dish or well (35-mm glass bottom dish or 6-well plates) and then treated with 200 μmol/L PA for 18 hours. After incubation, cells were washed and rinsed for further analysis by immunocytochemistry or immunoblotting. For immunocytochemistry, cells were incubated with 2.5% normal horse serum for 40 minutes before incubation with 200 μmol/L PA for 18 hours. After incubation at 4°C overnight, cells were washed 2 times with phosphate-buffered saline-Tween for 10 minutes each and 1 time with phosphate-buffered saline for 15 minutes, followed by staining using an ABC-peroxidase kit (Vector Laboratories Inc, Burlingame, CA). To examine the role of JNK in the PA-induced lipotoxicity, cells were pretreated with dimethyl sulfoxide (DMSO) or a JNK inhibitor SP600125 (10 μmol/L) for 6 hours before the PA treatment in some experiments. To assess the impact of mTORC1, PERK, and oxidative stress on JNK phosphorylation, WT and SESN1/2/3 KD HepG2 cells were pretreated with rapamycin (20 nmol/L), GSK2656157 (2 μmol/L), and N-acetyl cysteine (5 mmol/L) for 2 hours before incubation with 200 μmol/L PA for 24 hours.

**Table 1. Continued**

| Gene | Species | Primer sequence (forward and reverse) | Application |
|------|---------|--------------------------------------|-------------|
| Ppia | Mouse   | 5'–CAG CTT TCA GTC GGA ACT CAG-3' 5'–ATC GAA TAG CAG GGA AGA-3' | qPCR         |
| Floxed Sesn1 | Mouse | 5'–GAAATGACGAGGCTGAACTG-3' 5'–CAATAGGAGGGACACCCGAC-3' | Genotyping   |
| Sesan2 knockout | Mouse | 5'– GGTCAGAGAAGGTCATAGGA-3' 5'–CCAAACCTTCTCCCTCATAC-3' 5'–CTCCACGGCCCTGTTTTA-3' | Genotyping   |
| Floxed Sesn3 | Mouse | 5'– GTTGTGCAAACCACATCAG-3' 5'–CTACAGGGTTATGTTATGCTATGAT-3' | Genotyping   |
| SESN1 sgRNA | Human | 5'– ATTCCTCAGGACTAGGACA-3' | CRISPR/Cas9 |
| SESN2 sgRNA | Human | 5'– CAGGGTGCTACTGCAGCCAGC-3' | CRISPR/Cas9 |
| SESN3 sgRNA | Human | 5'– AGCTGCTACAGACATCACTGACCT-3' | CRISPR/Cas9 |

**Gene Expression Omnibus Data and Statistical Analysis**

Human NASH liver microarray data were downloaded from Gene Expression Omnibus (Accession #GSE48452), and SESN1/2/3 gene expression was analyzed. Statistical analysis was performed using Welch’s unpaired two-tailed t test between 2 groups and one-way or two-way analysis of variance followed by Tukey post hoc tests for multiple groups (GraphPad Prism 9.1.0; San Diego, CA).
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