

## The Role of Fatty Acid Metabolism and Apolipoproteins in Ths-Induced Hepatic Steatosis in Mice

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### Abstract

**Background:** Hepatic steatosis results from the increase of accumulation of lipids in the liver, decrease of beta fatty acid oxidation and/or decrease in the export to peripheral tissue by apolipoproteins. Previously, we showed that third hand smoke (THS) toxins result in hepatic steatosis in mice.

**Objective:** The goal of this paper was two-fold: (1) To determine whether THS toxins alter key molecules involved in beta fatty acid metabolism and (2) to determine whether the levels of apolipoprotein B is decreased in THS-exposed mice leading to decrease export of lipid from the liver.

**Methodology:** Mice were exposed to THS toxins for 6 months before performing the studies shown here. THS-exposed mice were also placed on western diet (WD) for five months or treated with AICAR to determine how THS-toxins affect the lipid metabolism of these animals.

**Results:** THS-exposed mice do not show significant difference in the levels of key fatty acid metabolism enzymes (CPT1, ACC, IDH2 and LCAD) compared to the control, suggesting THS toxins do not decrease the levels of these enzymes. THS-exposed mice have lower levels of SIRT3 and ATP. These mice also have lower IDH2 activity. THS-exposed mice also have lower levels of apolipoprotein B compared to control, suggesting the excess fatty acids, which are converted to TG in the liver, are not being transported to peripheral tissue for usage or storage.

**Conclusion:** These results suggest that even though THS toxins do not alter the levels of fatty acid metabolism enzymes, exposure result in lower levels of SIRT3 and lower IDH2 activity resulting in lower production of ATP in THS-exposed mice. THS toxins exposure also decrease of transport of lipids out of the liver by decreasing the levels of apolipoprotein B. Consequently, THS-exposed mice have an increase in lipid accumulation in the liver resulting in hepatic steatosis.

**Keywords:** Lipid transport; VLDL assembly; Oxidation of lipids; Toxicology, Energy imbalance

### Introduction

The liver is one of the largest and most metabolically active organs in the body. In addition to performing a myriad of metabolic functions that regulate energy levels, the liver is a major site for lipid and protein synthesis and is responsible for the delivered to other tissues [1,2]. Under normal conditions, the liver is constantly regulating synthesis and breakdown of lipids to meet physiological metabolic demands. In the liver, AMPK and SREBP1c regulate the synthesis of lipids in the liver. When there is an excess of fatty acids in the liver, SREBP1c is activated and activates acetyl-coA carboxylase (ACC), which leads to the activation of malonyl CoA, resulting in the synthesis of triglycerides (TGs) [3-7]. If not secreted into circulation or metabolized these TGs accumulate in the liver and can result in hepatic steatosis. Conversely, the liver can increase breakdown of fatty acids via beta-oxidation to decrease lipid accumulation by decreasing the activation of SREBP1c and increasing the activation of key mitochondrial enzymes that participate in the oxidation of fatty acids, such as carnitine palmitoyltransferase I (CPT1), long-chain acyl-CoA

dehydrogenases (LCAD), and isocitrate dehydrogenase 2 (IDH2). These enzymes catalyze fatty acid breakdown starting by transporting the activated fatty acids into the mitochondria by CPT1. LCAD then further processes the fatty acids that enter the tricarboxylic acid (TCA) cycle in the form of acetyl-CoA. The processing of the fatty acids continues via the participation of IDH2, which allows the fatty acids to be converted into high-energy molecules that can be used for the synthesis of ATP during oxidative phosphorylation. Therefore, low ATP production can be a result of a decrease in levels or function of LCAD or IDH2 [8-13]. Perturbation of this catabolic pathway can result in abnormal fatty acid metabolism, which can consequently lead to the accumulation of lipids in the hepatocytes, resulting in hepatic steatosis.

Another path by which the hepatocytes can further control lipid accumulation is by transporting excess lipids out of the liver by increasing very low density lipoprotein (VLDL) assembly and secretion. A decrease in VLDL assembly and secretion has been associated with hepatic steatosis [14-16]. VLDL particles, which are synthesized in the liver, are responsible for delivering TGs to the different cells of the body. VLDL particles are composed of TG, cholesterol and apolipoprotein (APOB). In the liver, VLDL synthesis is greatly affected by the levels of microsomal triglyceride transfer protein

(MTTP) and APOB [17-25]. APOB in particular has been associated with non-alcoholic fatty liver (NAFLD). Past studies have shown that insulin has the ability to inhibit the expression of APOB, whereas fatty acids induce its expression [19,20]. Also, patients with NAFLD have reduced expression of APOB, suggesting that their liver is unable to transport TG into peripheral tissue, resulting in accumulation of lipids in the hepatocytes [17]. The function of APOB is affected by MTTP proteins, which allow APOB to be released from the ER and start participating in the organization of the VLDL particles. Low levels of MTTP have also been associated with the degradation of APOB and the decrease of VLDL particles [22-25]. Therefore, altering the level of MTTP and APOB in the liver affects the transport of lipids out of the liver. Consequently, lipid accumulation results in hepatic steatosis.

The ability of the liver to process fatty acids and transport lipids to other tissues is affected by toxins, diet and drugs [26-34]. Previously, we have shown that exposure to THS toxins results in lipid accumulation in hepatocytes of exposed mice. Recently, we have shown that these mice also have high levels of oxidative stress that lead to lung and liver damage as well as abnormal cognitive behavior [33]. We also showed that THS toxins result in altering the SIRT1/AMPK/SREPB1c pathway [34]. Modulating the levels of oxidative stress with APAP (Tylenol) and antioxidants resulted in worsening and improvement of hepatic steatosis, respectively, in mice exposed to THS toxins. These findings suggested that THS toxins result in hepatic damage by altering the oxidative stress levels and increasing lipid synthesis. The goal of this study was to determine whether THS also affects fatty acid metabolism and lipid transport. Our results show that THS toxins decrease the activity of IDH2 leading to decrease in lipid breakdown and lower ATP production. Furthermore, THS toxins also decrease the levels of MTTP and APOB in the liver suggesting THS exposure also targets lipid transport. Our findings show that THS toxins affect the metabolism fatty acids and transport of lipids in mice highlighting the danger of THS toxins for liver health.

## Materials and Methods

### Animals

All male C57BL/6 mice used for these experimental procedures were housed under the following environmental conditions: 12 h light/dark cycle in convectional cages with ad libitum. The regular standard chow diet was composed of 58% carbohydrates, 28.5% protein, 13.5% fat and water.

### Ethics statement

The experiments were done according to animal protocols that were approved by the University of California, Riverside, Institutional Animal Care and Use Committee (UCR-IACUC). The mice used for these studies were euthanized by carbon dioxide (CO<sub>2</sub>) inhalation, which is the most common method of euthanasia used by NIH. The amount and length of CO<sub>2</sub> exposure were approved by UCR-IACUC.

### THS exposure method

The THS exposure method used for these studies has been previously described in detail. C57BL 6J mice were housed in THS-exposed material to study the effects of toxins in liver health for a period of 24 weeks. THS exposure of the material (10 g curtain composed of cotton, 10 g upholstery composed of cotton and 10 g carpet composed mainly of fiber) was done using a Teague smoking

apparatus. The machine was set to smoke two packs of 3R4F research cigarettes five days a week to model a regular smoker (cigarette/15 min). The total particulate matter (TPM) was set to 30 µg/m<sup>3</sup>, which is the TPM found in the homes of smokers. The mice were housed in the THS-exposed material for a week and the material was exposed for four weeks to allow the toxins to age. After four weeks the material was discarded. The control C57BL 6J mice were not exposed to THS toxins and were housed in a different room than the THS-exposed mice.

### Western diet treatment

After weaning, normal C57 BL/6J male mice and Third-Hand Smoke (THS)-exposed C57 BL/6J male mice were placed on a western chow diet for duration of 6 months. The western diet was purchased from Harlan laboratories (Cat# TD88137) and was composed of 21% anhydrous milk fat (butterfat), 34% sucrose, and 0.2% cholesterol.

### AICAR treatment

AICAR was purchased from Toronto Research Chemicals (Cat# A440500) and was dissolved in phosphate buffered saline (PBS). THS-exposed or control mice were given an intraperitoneal injection (I.P) of either AICAR (250 mg/kg for 5 days/week) or a comparable volume of PBS for eight weeks.

### Tissue extracts

After six months of THS exposure, the livers of THS-exposed male mice were extracted and immediately frozen and stored at -80°C. Protein extracts were made from portions of the frozen tissue unless otherwise specified by the kits' instruction manuals, and were used for ELISA analysis. Liver tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA), centrifuged, and the supernatant collected unless otherwise specified by the kits' instruction manuals.

### Blood extracts

Blood was extracted directly from the heart and allowed to coagulate for 20-30 min. The samples were then centrifuged at 2000 RPM to induce phase separation between plasma proteins and serum. The serum was then collected from the blood samples. The serum was used immediately for assays or immediately frozen and stored at -80°C.

### Total lipid

Lipid content in the liver was determined using the Folch method [7]. Tissue was homogenized with chloroform/methanol (2/1) to a final volume 20 times the volume of the tissue sample and agitated for 15-20 min. The homogenate was centrifuged to recover the liquid phase. The solvent was washed with 0.2 volume of 0.9% NaCl solution. After vortexing, the mixture was centrifuged at 2000 RPM to separate the two phases. The upper phase was removed and the lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator. To determine the total amount lipids in the sample the weight of the vial was subtracted from the weight of the vial plus lipids.

### Measurement of fatty acids (FA) levels

Fatty acids were quantified using Abcam kits (Cat #ab65341). In this assay, FAs are converted to their CoA derivatives and subsequently

oxidized, leading to formation of color/fluorescence. Fatty acid levels were quantified using the absorbance values obtained from the liver tissue homogenates samples at 550 nm.

### Measurement of triglyceride (TG) levels

The TG contents were measured using Cayman Triglyceride Colorimetric Assay Kit (Cat# 10010303). The Triglyceride Colorimetric Assay uses the enzymatic hydrolysis of triglycerides by lipase to produce glycerol and free fatty acids. The absorbance of standards and samples were read at 550 nm. Absorbance values were used to generate a standard curve. The levels of TG were interpolated from this standard curve. Measurement of IDH2 levels: The IDH2 levels in liver of THS-exposed mice were quantified using IDH2 ELISA kit from Mybiosource (Cat# MBS074031). IDH2 levels were quantified by incubation of tissue homogenate in an anti-IDH2 antibody coated plate, followed by an HRP conjugated secondary antibody. The optical density (O.D.) of the samples was then read at 450 nm. The values were used to quantify the levels of IDH2 in each sample.

### Measurement of LCAD levels

The LCAD levels in THS-exposed mice were quantified using the ELISA kit from Mybiosource (Cat# MBS931062). LCAD levels were quantified by incubation of tissue homogenate in an anti-LCAD antibody coated plate, followed by an HRP conjugated secondary antibody. The optical density O.D. of the plate was then read at 450 nm. The values were used to quantify the levels of LCAD in the liver.

### Measurement of CPT1 levels

The CPT1 levels of THS-exposed mice were quantified using CPT1 ELISA kit from Mybiosource (cat# MBS7252964). CPT1 levels were quantified by incubation of tissue homogenate in an anti-CPT1C antibody coated plate followed by detection by substrate solution provided in the kit. The O.D. of the plate was then read at 450 nm. The values were used to quantify the levels of CPT1 in each sample.

### Measurement of ACC levels

ACC levels were determined using an ELISA kit from Mybiosource (Cat# MBS2606160). ACC levels were quantified by incubation of liver tissue homogenate in an anti-ACC antibody coated plate, by detection by substrate solution provided in the kit. The O.D. of each well was measured at 450 nm. The resulting O.D. values were used to calculate the ACC levels in the liver.

### Measurement of APOB levels

The APOB levels in liver of THS-exposed mice were quantified using APOB ELISA kit from Neoscientific (Cat# MA0520). APOB levels were quantified by incubation of tissue homogenate in an anti-APOB antibody coated plate. The O.D. of the plate was then read at 450 nm. The values were used to quantify the standard curve, which was used to calculate the levels of APOB in the liver samples.

### Measurement of MTTP levels

MTTP levels in the livers of THS-exposed mice were quantified using MTTP ELISA kit from Mybiosource (Cat# MBS9329329). MTTP levels were quantified by incubation of tissue homogenate in an anti-MTTP antibody coated plate, followed by an HRP conjugated

secondary antibody. The optical density O.D. of the plate was then read at 450 nm. The values were used to generate a standard curve and to quantify the levels of MTTP in the liver.

### Measurement of VLDL levels

VLDL levels in the livers of THS-exposed mice were quantified using VLDL ELISA kit from Mybiosource (Cat# MBS706037). VLDL levels were quantified by incubation of tissue homogenate in an anti-VLDL antibody coated plate, followed by an HRP conjugated secondary antibody. A standard curve was generated and used to quantify VLDL levels in liver tissue of THS-exposed mice and control mice.

### Statistical analysis

For the statistical analysis of experiments mentioned above, we used Graphpad InStat Software (Graphpad, La Jolla, CA, USA). Statistical comparisons between two-groups were performed using the unpaired Student's t-test. All data are mean $\pm$ SD represented by the error bars. Means among samples were considered significantly different when  $p < 0.05$ .

### Results

Hepatic steatosis can result from an increase of de novo lipid synthesis, decrease of breakdown of fatty acids, or decrease of the transport of lipids out of the liver. Previously, we showed that THS toxins increase oxidative stress in the liver of mice resulting in hepatic steatosis [34]. In this study we investigated whether THS-exposure changes the levels and/or activity of key enzymes involved in the breakdown of fatty acids and in the transport of lipids out of the liver.

### THS-exposure affects FA, TG and total lipid levels in the liver

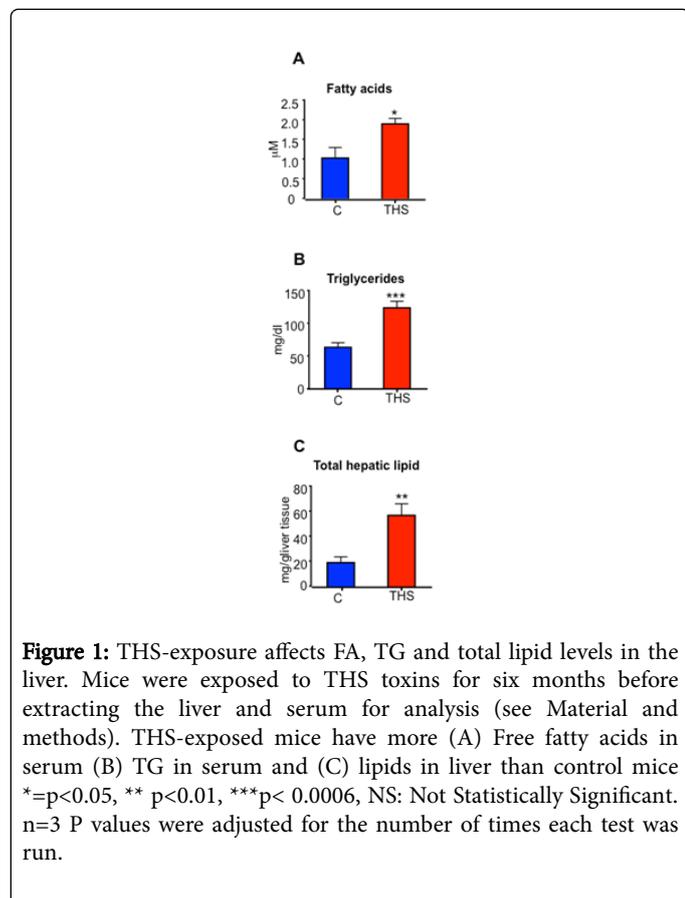
Quantification of fatty acids, TG and lipids in THS-exposed mice showed higher levels of all three in the liver of THS-exposed mice (Figures 1A-1C). Because western diet (WD), which is rich in fat (see Materials and Methods section) and has been shown to decrease the activation of AMPK, an enzyme that regulates the breakdown of fatty acids in the liver [35], we tested whether WD augments the effects caused by THS-exposure. Mice were placed at weaning (3 weeks of age) on WD in conjunction with THS exposure for 5 months. At the end of this period we analyzed again for FA, TG and total hepatic lipids and found that none of these parameters were significantly higher when compared to the mice on the chow diet and exposed to THS (Figures 2A-2C). However, there was a significant difference in the levels compared to those of mice feed WD only (Figures 2A-2C).

To investigate whether a stimulator of AMPK phosphorylation, 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) [35], resulted in improved fatty acid metabolism in the THS-exposed mice, we treated mice exposed to THS for 6 months with AICAR at 250 mg/kg for 5 days/week for eight weeks via an intraperitoneal injection and treated the controls in a similar way with comparable volume of PBS. Indeed, we found that AICAR was able to reverse the adverse effects of THS exposure in lipid metabolism. The levels of fatty acids, TG and total lipids were returned to normal levels even in the presence of THS exposure (Figures 3A-3C).

### Effects of THS-exposure on the levels/activity of enzymes involved in fatty acid metabolism

Based on the results presented above we examined whether THS-exposure affects the levels of key enzymes that play a role in the breakdown of fatty acids in the liver. For these studies we measured the levels of Acetyl-CoA carboxylase (ACC), carnitine palmitoyltransferase I (CPT1), long-chain acyl-CoA dehydrogenases (LCAD), and isocitrate dehydrogenase 2 (IDH2) after six months of exposure.

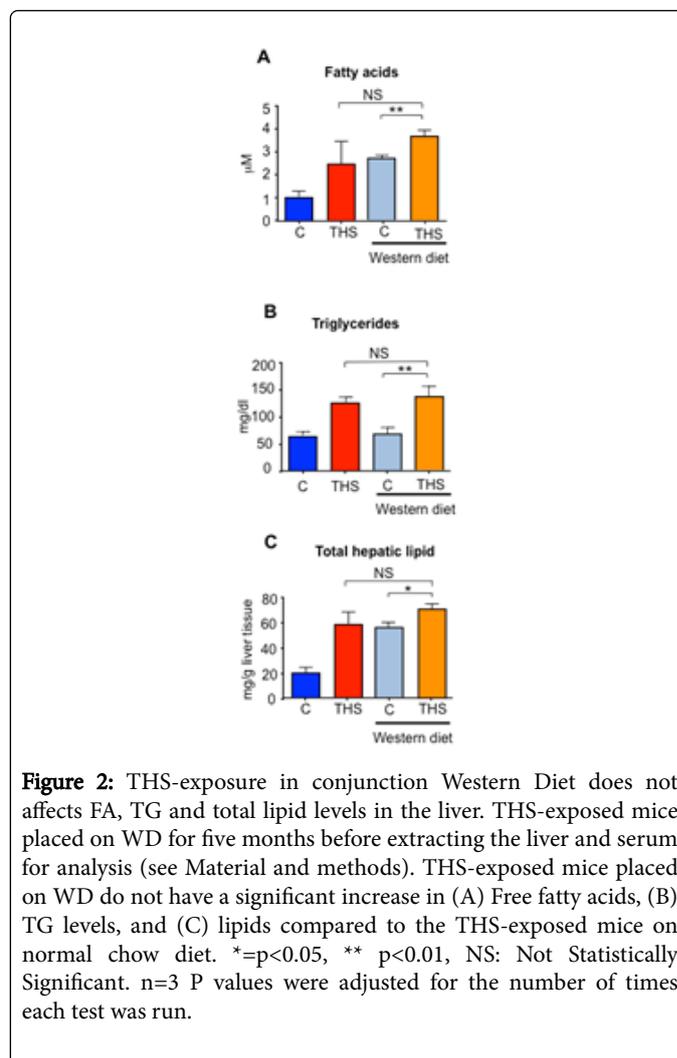
Except for IDH2, that we found to have a tendency to be decreased, the other three enzymes did not show levels different from the control and the THS-exposed mice (Figures 4A-4D). Because, we observed that IDH2 levels were consistently lower in THS-exposed mice although be it not quite significantly lower, we tested for activity of IDH2 and found that its activity is significantly decreased in THS-exposed mice (Figure 5A).



**Figure 1:** THS-exposure affects FA, TG and total lipid levels in the liver. Mice were exposed to THS toxins for six months before extracting the liver and serum for analysis (see Material and methods). THS-exposed mice have more (A) Free fatty acids in serum (B) TG in serum and (C) lipids in liver than control mice \*= $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\* $p < 0.0006$ , NS: Not Statistically Significant.  $n=3$  P values were adjusted for the number of times each test was run.

Because it is known that SIRT3 plays a key role in fatty acid metabolism and that it modulates the activity of IDH2, we examined the levels of SIRT3 in THS-exposed mice.

We found lower levels of SIRT3 in the liver of THS-exposed mice when compared to the control suggesting that these mice might have lower fatty acid breakdown than the controls (Figure 5B). Because IDH2 is an important enzyme in the TCA cycle, which is key for the production of the reduction potential in the cell (NADPH and FADH<sub>2</sub>), which then provides the electrons to the oxidative phosphorylation chain to produce ATP, we measured the level of ATP to determine whether it was affected by THS exposure.

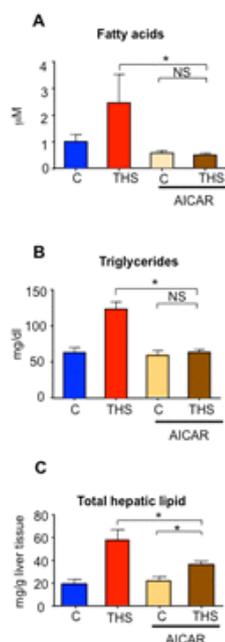


**Figure 2:** THS-exposure in conjunction Western Diet does not affect FA, TG and total lipid levels in the liver. THS-exposed mice placed on WD for five months before extracting the liver and serum for analysis (see Material and methods). THS-exposed mice placed on WD do not have a significant increase in (A) Free fatty acids, (B) TG levels, and (C) lipids compared to the THS-exposed mice on normal chow diet. \*= $p < 0.05$ , \*\*  $p < 0.01$ , NS: Not Statistically Significant.  $n=3$  P values were adjusted for the number of times each test was run.

We found that ATP levels were significantly reduced in the liver of THS-exposed mice (Figure 5C). Taken together these results suggest that the capacity of lipids to undergo oxidation in the liver is reduced by THS-exposure, which very likely leads to the accumulation of lipids in the hepatocytes.

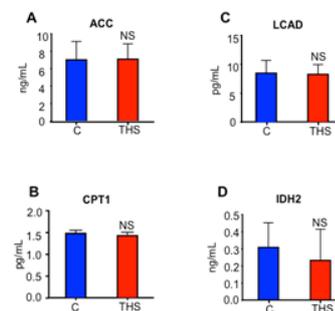
### THS exposure results in a decrease of VLDL assembly in the liver

To determine whether THS-exposure affects lipid transport out of the liver, we investigated whether THS toxins alter the level of proteins involved in lipid transport out of the liver. Both apolipoprotein B (APOB) and microsomal triglyceride transfer protein (MTTP) have been associated with the transport of lipids out of the liver into peripheral tissues such as to the adipose tissue. We focused on APOB and MTTP because these proteins play a key role in VLDL assembly, a particle type that is critical in transport of lipids out of the liver [23-26].



**Figure 3:** THS-exposure in conjunction with AICAR treatment affects FA, TG and total lipid levels in the liver. THS-exposed mice treated with AICAR for eight weeks before the liver and serum was extracted for analysis. THS-exposed mice treated with AICAR have less (A) Free fatty acids (B) TG levels and (C) lipids than THS-exposed mice not treated with AICAR. \*= $p < 0.05$ , \*\*  $p < 0.01$ , NS: Not Statistically Significant.  $n=3$  P values were adjusted for the number of times each test was run.

THS-exposed mice showed lower levels of APOB (Figure 6A) and MTTP (Figure 6B) in the liver compared to the controls. In addition, we also found that THS-exposed mice had lower VLDL levels than controls (Figure 6C). These findings indicate that THS toxins alter processes involved in lipid transport out of the liver through decreased levels of VLDL particle formation and consequently this contributes to lipid accumulation in the liver of THS-exposed mice.



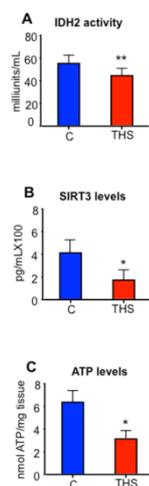
**Figure 4:** Effects of THS-exposure on the levels of enzymes involved in fatty acid metabolism. Mice were exposed to THS toxins for six months before extracting the liver for analysis of enzyme levels (see Material and methods). (A-B) THS-exposed mice also have no significant change in acetyl-coA carboxylase (ACC) and carnitine palmitoyltransferase I (CPT1). (C) THS-exposed mice do not have lower long-chain acyl-CoA dehydrogenases (LCAD) levels or lower isocitrate dehydrogenase 2 (IDH2) levels than controls (D). \*= $p < 0.05$ , \*\*  $p < 0.01$ , NS: Not Statistically Significant.  $n=3$ . P values were adjusted for the number of times each test was run.

## Discussion

We have previously shown that THS exposure results hepatic steatosis in mice [3]. More recently we showed that, THS-exposed mice have high levels of hepatic oxidative stress leading to liver damage. We also showed that the lipid accumulation observed in THS-exposed mice is due to increase in the levels of oxidative stress, primarily of ROS, and inhibition of the SIRT1/AMPK/SREBP1c pathway [34]. The goal of this study is to determine whether THS toxins affect fatty acid metabolism and lipid transport resulting in THS-induced hepatic steatosis.

In this study, we show that THS-exposed mice are unable to metabolize and transport lipids efficiently compared to the control mice. These mice have higher amount of lipids, fatty acids and TG suggesting these animals do not have the metabolic efficiency exhibited by the control mice. Western diet (WD) has been associated with an increase in lipid accumulation in the liver whereas AICAR treatment has been associated with a decrease in lipid accumulation [35-40]. WD has been shown to increase oxidative stress and increase fat accumulation in the liver by decreasing fatty acid metabolism resulting in hepatic steatosis. THS-exposed mice placed on WD did not have a worse lipid profile than the THS-exposed mice placed on chow diet. However, AICAR treatment led to improved lipid metabolism in THS-exposed mice.

THS-exposed mice treated with AICAR had lower FA, TG and hepatic lipids. These finding suggest AICAR is able to reverse the lipid abnormalities induced by THS toxins but WD is unable to further worsen the lipid abnormalities triggered by THS toxins.

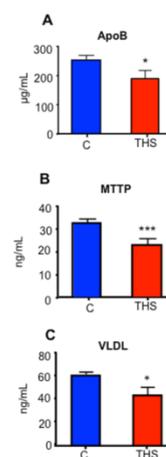


**Figure 5:** THS toxins decrease IDH2 activity, SIRT3 and ATP levels in the liver of THS-exposed mice. Mice were exposed to THS toxins for six months before extracting the liver for analysis of enzyme activity and levels (see Material and methods). (A) THS-exposed mice have lower IDH2 activity than control mice. (B) THS-exposed mice have lower SIRT3 levels and ATP levels (C) than control mice. \* =  $p < 0.05$ , \*\*  $p < 0.01$  NS = Not statistically significant.  $n = 10$  for A and  $n = 6$  for B and C. P values were adjusted for the number of times each test was run.

Fatty acid metabolism plays a key role in the development of hepatic steatosis [32]. In our previous study we showed THS-toxins increase the oxidative stress levels and lower SIRT1 levels [34]. Consequently, THS-exposed mice have greater lipid synthesis than the control mice.

In this study we focused on SIRT3 because it is a key regulator of fatty acid breakdown. SIRT3 plays a key role in lipid homeostasis in the liver by increasing fatty acid metabolism in the mitochondria. This sirtuin also regulates fatty acid metabolism by enhancing the activity of IDH2, which is involved in processing the fatty acids via the TCA cycle in the liver [11,12]. THS-exposed mice show lower levels of SIRT3 as well as lower activity of IDH2 suggesting that THS toxins alter lipid metabolism by also targeting the breakdown of fatty acids in the mitochondria. Consequently, THS-exposed mice do not have the same metabolic efficiency as the controls; they have less fatty acid processing and more lipid accumulation in the liver than controls.

Hepatic steatosis can also result from abnormal lipid transport. We were able to show that THS toxins result in a decrease in the levels of APOB and MTTP, two key molecules involved in the assembly of VLDL particles. Apolipoproteins play a key role in transporting lipids from one organ to another. APOB plays a key role in the transport of lipids out of the liver to peripheral tissue [18-26]. Decreasing the levels of this protein results in the decrease of transport of lipids out of the liver and consequently in the accumulation of lipids in the liver. Insulin has been associated with the negative regulation of APOB in the liver. Previously we have showed that THS-exposed mice have insulin resistance and high levels of insulin in the serum. Therefore it is possible that insulin resistance contributes to the low levels of APOB in THS-exposed mice.



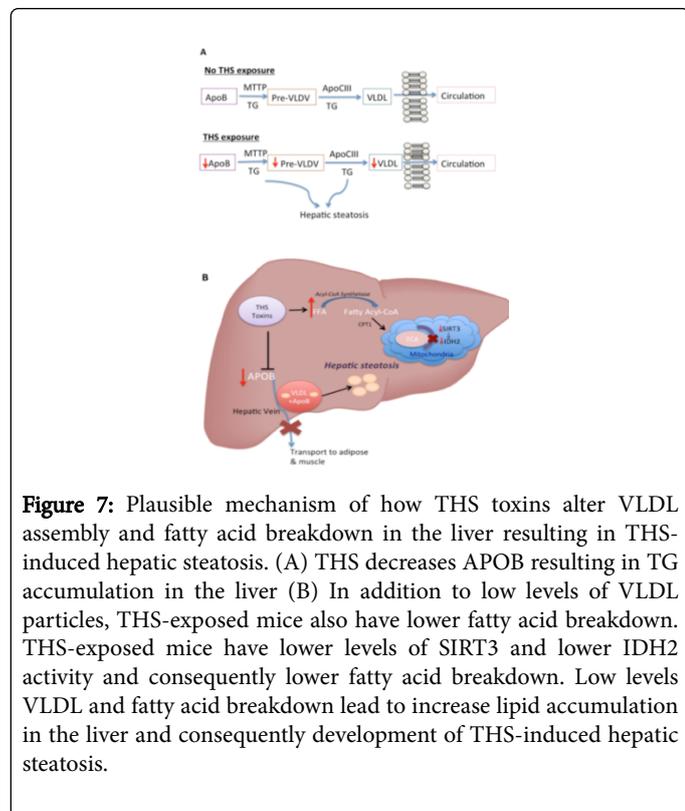
**Figure 6:** THS exposure results in a decrease of VLDL assembly in the liver. Mice were exposed to THS toxins for six months before extracting the liver for analysis of enzyme levels (see Material and methods). (A) THS-exposed mice have lower levels of APOB in the liver than control mice. (B) THS toxins also result in lower MTTP protein in the liver of THS-exposed mice. (C) Lastly, THS-exposed mice also have lower levels of VLDL in the liver compared to the control mice. \* =  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0006$ , NS = Not statistically significant.  $n = 6$  P values were adjusted for the number of times each test was run.

Some of the current treatment strategies available for hepatic steatosis are diet, lifestyle changes and taking medication. We have previously shown that THS-exposed mice treated with antioxidants had a decrease in the levels of hepatic lipids suggesting that antioxidants improve THS-induced hepatic steatosis [34]. Our findings presented in this paper show that THS toxins also affect fatty acid metabolism by decreasing SIRT3 levels and VLDL assembly in the liver of THS-exposed mice. Taken together these findings suggest THS-induced hepatic steatosis could be treated with activators of SIRT3, AICAR or antioxidants.

SIRT3 is known to play a key role in the modulation of oxidative stress, which we know plays a key role in the development of THS-induced hepatic steatosis [34] and therefore activating SIRT3 with a natural compound or drug can be a potential treatment for THS-induced hepatic steatosis. The activation of SIRT3 will result not only in the breakdown of fatty acids via TCA cycle but also result in the decrease of oxidative stress making this molecule a great target. Another potential treatment for THS-induced hepatic steatosis is AICAR. We have shown that when THS-exposed mice were treated with AICAR the levels of TG and fatty acids were decreased. Additional studies need to be done to elucidate whether these treatment strategies (activators of SIRT3, AICAR or antioxidant or both) could be used to treat humans with THS-induced hepatic steatosis.

In conclusion, THS toxins cause lipid accumulation in the liver by targeting proteins involved in VLDL assembly (Figure 7A). THS toxins also decrease SIRT3 levels and IDH2 activity in the liver leading to a decrease in fatty acid metabolism (Figure 7B). In addition to a lower breakdown of fatty acids, THS-exposed mice also have lower VLDL levels compared to the control mice. These findings are significant

because they provide further characterization of THS-induced hepatic steatosis in mice and further insights into the danger of exposure of THS toxins to liver health.



**Figure 7:** Plausible mechanism of how THS toxins alter VLDL assembly and fatty acid breakdown in the liver resulting in THS-induced hepatic steatosis. (A) THS decreases APOB resulting in TG accumulation in the liver (B) In addition to low levels of VLDL particles, THS-exposed mice also have lower fatty acid breakdown. THS-exposed mice have lower levels of SIRT3 and lower IDH2 activity and consequently lower fatty acid breakdown. Low levels VLDL and fatty acid breakdown lead to increase lipid accumulation in the liver and consequently development of THS-induced hepatic steatosis.

## Acknowledgments

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