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Activation of PPAR α ameliorates hepatic insulin resistance and steatosis in high fructose-fed mice despite increased ER stress

Running Title: PPAR α activation eliminates hepatic insulin resistance against increased ER stress

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ABSTRACT

Endoplasmic reticulum (ER) stress is suggested to cause hepatic insulin resistance by increasing *de novo* lipogenesis (DNL) and/or directly interfering with insulin signaling via activation of the JNK and IKK pathway. The present study interrogated these two proposed mechanisms in a mouse model of hepatic insulin resistance induced by a high fructose (HFru) diet with the treatment of fenofibrate (FB, 100 mg/kg/day), a PPAR α agonist known to reduce lipid accumulation while maintaining elevated DNL in the liver. FB administration completely corrected HFru-induced glucose intolerance, hepatic steatosis and the impaired hepatic insulin signaling (pAkt and pGSK3 β). Intriguingly, both IRE1/XBP1 and PERK/eIF2 α arms of the UPR signaling were activated. While retaining the elevated DNL (indicated by the upregulation of SREBP1c, ACC, FAS and SCD1 and $^3\text{H}_2\text{O}$ incorporation into lipids), FB treatment markedly increased FA oxidation (indicated by induction of ACOX1, pACC, β -HAD activity and ^{14}C -palmitate oxidation) and eliminated the accumulation of diacylglycerols (DAGs) which is known to impact on insulin signaling. Despite the marked activation of UPR signaling, neither JNK nor IKK appeared to be activated. These findings suggest that lipid accumulation (mainly DAGs), rather than the activation of JNK or IKK is pivotal for ER stress to cause hepatic insulin resistance. Therefore, by reducing the accumulation of deleterious lipids, activation of PPAR α is able to ameliorate hepatic insulin resistance against increased ER stress.

(Word count 222)

Key words: PPAR α activation, ER stress, lipid synthesis, fatty acid oxidation, JNK/IKK, hepatic insulin signaling

INTRODUCTION

Liver is one of the most metabolically active and insulin responsive organs regulating glucose homeostasis, lipid metabolism and protein synthesis (1). Under normal conditions, insulin suppresses hepatic glucose production from glycogenolysis and gluconeogenesis while promotes glucose storage in the form of glycogen to help control postprandial glucose level. However, the ability of insulin to shut down glucose production from the liver is diminished under the state of hepatic insulin resistance which in turn leads to the manifestation of hyperglycaemia (2). Although the pathogenesis of hepatic insulin resistance is likely to be multi-factorial, increased endoplasmic reticulum (ER) stress and an accumulation of lipids within the liver have been demonstrated to be important mechanisms (3; 4).

Lipid accumulation in the liver or hepatic steatosis can lead to insulin resistance by interfering with the insulin signal transduction through lipid metabolites such as diacylglycerols (DAGs) and ceramide (4). Hepatic steatosis can result from increased FA influx, elevated *de novo* lipogenesis (DNL) and/or reduced fatty acid (FA) oxidation (1; 4). In humans, elevated DNL from the increased consumption of sucrose is the predominant mechanism for the development of hepatic steatosis with fructose (breakdown product of sucrose) being the major culprit (5; 6).

Recent studies in animal models (7; 8) have identified a possible role of ER stress in the development of hepatic insulin resistance during elevated DNL. When ER stress occurs, the ER mounts the unfolded protein response (UPR) which involves the activation of three major branches of signal transducers: inositol-requiring protein 1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (9). Activation of these canonical mechanisms is crucial for cellular adaption and resolution of ER stress. However, chronic activation of UPR signaling has been demonstrated to activate c-jun N-terminal kinase (JNK) and I κ B kinase (IKK).

The IRE1 branch of the UPR can activate JNK (10) and IKK (11) by forming a complex with the tumor-necrosis factor- α -receptor-associated factor 2 (TRAF2). Meanwhile, the PERK/eIF2 α branch has also been reported to be capable of activating JNK (12). As activated JNK (8; 13) and/or IKK (14) can directly serine/threonine phosphorylate insulin-receptor-substrate (IRS) leading to the inhibition of insulin signaling transduction, it has been suggested that JNK and IKK are the key molecules linking activated UPR and hepatic insulin resistance (15).

More recently, we have found that elevated DNL and insulin resistance in the liver of high fructose (HFru) fed mice is coupled with activation of the IRE1 and PERK branches of the UPR (16). However, it is unclear whether hepatic insulin resistance results from lipid accumulation or activated JNK/IKK pathways during increased ER stress. Interestingly, hepatic DNL is increased by the activation of PPAR α (17; 18) which has also been shown to reverse hepatic steatosis (19; 20). Because ER stress is tightly associated with DNL, we hypothesized treatment of HFru-fed mice with a PPAR α agonist would activate both the IRE1 and PERK branches while preventing hepatic steatosis. Under these conditions, we would then be able to interrogate the implication of these two mechanisms (lipid accumulation or activated JNK/IKK) in hepatic insulin resistance in the face of increased ER stress. Our results showed that accumulation of lipids namely DAGs, rather than the activation of JNK or IKK, is the key factor of hepatic insulin resistance during increased ER stress. Activation of PPAR α with FB is able to eliminate hepatic insulin resistance during HFru feeding by reducing DAG levels despite the presence of ER stress evidenced by the dual activation of the IRE1/XBP1 and PERK/eIF2 α pathways.

MATERIAL AND METHODS

Animals

Male C57BL/6J mice (14 weeks) from the Animal Resources Centre (Perth, Australia) were kept at $22\pm 1^\circ\text{C}$ on a 12-h light/dark cycle. After 1 week of acclimatization, mice were fed *ad libitum* for up to 15 days with either a chow (CH), fructose-rich (HFru) diet. CH diet consisted of 70% calories from starch, ~10% calories from fat, and ~20% calories from protein (Specialty Feeds, Glen Forrest, Western Australia), HFru diet (35% fructose, 35% starch, ~10% fat and ~17% protein). The detailed recipe for the HFru diet is described in our previous studies (16; 21). FB (Sigma-Aldrich, Australia) was supplemented to the animal by mixing into the diets at a concentration of 100mg/kg/day. All experiments were approved by the Animal Ethics Committees of the RMIT University (#1012).

Body weight and food intake were measured daily. The whole body metabolic rate was measured at 22°C using an indirect calorimeter (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments, OH, USA) as described previously (22) between 5-8 days after the administration of FB. Mice were fasted for 5-7 hours before being killed, tissues of interest were collected and freeze-clamped immediately. Epididymal fat mass was weighed using an analytical balance. Liver triglycerides were extracted by the method of Folch and determined by a Peridochrom Triglyceride GPO-PAP kit (Roche Diagnostics, Australia) as previously described (16). In separate experiments, glucose tolerance tests (GTT; 2.5g glucose/kg BW, *ip*) were performed on the 5-7 hours fasted mice using a glucometer (AccuCheck Proforma Nano; Roche, Victoria, Australia) and blood samples were collected at 0, 30 and 60 min for plasma insulin measurement. For the assessment of insulin signaling in the liver, the mice were injected with insulin (2U/kg BW, *ip*) 20 min before tissue collection.

Measurement of hepatic FA oxidation *de novo* lipogenesis (DNL)

FA oxidation was measured in liver homogenates using methods described previously (23). Briefly, the liver homogenate (50 μ l) was incubated at 30°C for 90 min in a reaction mixture (pH 7.4) containing (in mM) 0.2 [¹⁴C]-palmitate (0.5 μ Ci), 2 L-carnitine and 0.05 Coenzyme A \pm 0.02 etomoxir, a specific inhibitor of FAs oxidation in mitochondria (24). The reaction was stopped by ice-cold 1 M perchloric acid. CO₂ produced from the reaction was captured in 1 M NaOH. ¹⁴C counts in the acid-soluble fraction were combined with the CO₂ values to give the total palmitate oxidation rate. Hepatic DNL was determined by measuring the incorporation of [³H]-H₂O into triglyceride in the liver as described previously (16; 25).

Citrate synthase and β 3 – hydroxyacyl– CoA dehydrogenase (β -HAD) activity

Approximately 20 mg of frozen liver tissue was homogenized in 175 mM KCl and 1.98 mM EDTA containing buffer (pH 7.4) with a glass homogenizer before being subjected to three freeze-thaw cycles. Citrate synthase and β -HAD activities were determined as described previously (16; 21), using a Flexstation 3 plate reader (Molecular Devices, Sunnyvale, CA).

Western blotting

Liver and red quadriceps muscle samples were homogenized in ice-cold lysis buffer at pH 7.5 containing (in mM): 50 Tris, 150 NaCl, 1% Triton X-100, 10 NaP, 100 NaF, 2 Na₃VO₄, 1 EDTA, 1 EGTA and 10% glycerol supplemented with protease inhibitor cocktail and phosphates inhibitor cocktail (Sigma Aldrich Pty Ltd, Australia) and DL-dithiothreitol. Protein samples were then denatured in SDS sample buffer (125 mM Tris-HCl, pH 6.8, 50% glycerol, 2% SDS, 5% β -mercaptoethanol and 0.01% bromophenol blue). The insulin signal transduction was assessed by total- and phospho (Ser473)- Akt, total- and phospho (Ser219)- glycogen synthase kinase 3 β (GSK-3 β) (Cell Signaling, USA). Key lipogenic enzymes were by Western blotting using specific antibodies including sterol regulatory element-binding protein (SREBP-1c, Santa Cruz, USA),

phospho and total acetyl-CoA carboxylase (ACC, Cell Signalling, USA), fatty acid synthase (FAS, Cell Signalling, USA), stearoyl-CoA desaturase 1 (SCD-1, Cell signaling, USA). Oxidative capacity: acyl-coenzyme A oxidase 1 (ACOX1, Santa Cruz). ER stress: phospho (Thr980)- protein kinase RNA-like endoplasmic reticulum kinase (p-PERK, Cell signaling, USA), total- and phospho (Ser51)- eukaryotic translation initiation factor 2 α (eIF2 α , Cell signaling, USA), growth arrest and DNA damage-inducible protein 34 (GADD34, Cell signaling, USA), C/EBP homologous protein (CHOP, Santa Cruz, USA), and phospho (Ser724)- inositol-requiring kinase 1 (IRE1, Abcam, USA), X-box binding protein 1 (XBP1, Santa Cruz, USA), Activating transcription factor 6 α (ATF6 α , Santa Cruz, USA). Serine/threonine kinases: phospho- (Ser176/177) I κ B kinase α/β (IKK α /IKK β , Cell Signalling, USA), I κ B alpha (I κ B α), phospho- (Thr183/Tyr185) c-jun N-terminal kinase (JNK, Cell Signalling, USA). Immunolabeled bands were quantified by densitometry and representative blots are shown.

Analyses of hepatic lipids

Diacylglycerol (DAG) and ceramide were extracted and quantified according to the methods of Preiss et al. (26). Lipids were extracted from liver homogenates using chloroform: methanol: PBS+0.2% SDS (1:2:0.8). Diacylglycerol kinase and ³²P-labelled ATP (0.55 GBq/mmol cold ATP) were added to the lysates preincubated with cardiolipin/octylglucoside, and the reaction was stopped after 2.5 h by the addition of chloroform: methanol (2:1). Samples were spotted onto thin-layer chromatography plates and developed. ³²P-labelled phosphatidic acid and ceramide-1-phosphate were identified by autoradiography, dried, scraped from the thin-layer chromatography plate and counted in a liquid scintillation analyser (LS6500, Beckman Coulter Inc, USA).

Statistical analyses

Data are presented as means \pm SE. One-way analysis of variance was used for comparison of the relevant groups. When significant differences were found, the Turkey-Kramer multiple comparisons test was applied. Differences at $p < 0.05$ were considered to be statistically significant.

RESULTS

FB treatment normalises HFru feeding induced adiposity and improved glucose intolerance.

HFru-feeding resulted in significant increases in calorie intake (~38%), the whole body oxygen consumption (VO_2 , ~ 8%) and the respiratory exchange ratio (RER), body weight gain (1.2 g) and adiposity (67% in epididymal fat mass, $p < 0.05$) compared to untreated CH-fed animal (CH-Veh) (Table 1). In CH-fed mice, FB treatment had no significant effects on body weight gain, adiposity, caloric intake or RER except for a 14% increase in VO_2 ($p < 0.05$ vs CH-Veh). In HFru-FB-fed mice, FB significantly ($p < 0.05$ for all) increased VO_2 (8%), reduced the RER and completely diminished HFru-induced body weight gain and adiposity. FB lowered blood glucose and insulin levels in the HFru-fed mice (both $p < 0.01$ HFru-FB), hence an improved HOMA-IR index. The untreated HFru-fed mice displayed glucose intolerance (Figure 1A) compared to CH-Veh (Figure 1A and B). FB treatment completely normalized the glucose tolerance seen in the HFru-fed mice to the levels of the CH-fed mice and reduced the requirement for plasma insulin level (Figure 1C).

FB treatment restored hepatic insulin signal transduction in HFru-fed mice.

In skeletal muscle, the insulin stimulated phosphorylation of Akt (all $p < 0.001$ vs corresponding basal) was unaltered by diet or FB treatment (Figure S1). In contrast, HFru feeding blunted the insulin-stimulated phosphorylation of Akt (by 53%, $p < 0.01$ vs CH) and its downstream target GSK3 β (by 60%, $p < 0.001$ vs CH) in the liver which were fully restored by the treatment of FB (Figure 2A and Figure 2B). This indicated HFru feeding resulted in impairment of hepatic insulin

signaling and that treatment of FB was effective in restoring hepatic insulin sensitivity.

FB treatment normalised hepatic lipid accumulation.

Lipid accumulation in the liver (ie. hepatic steatosis) is believed to be closely linked to insulin resistance (1), we hence examined the effects of FB treatment on hepatic lipid content. As expected, the HFru feeding induced a marked increase in hepatic triglycerides (TG) levels (2.7-fold, $p < 0.01$ vs CH) which was ameliorated by the treatment of FB (Figure 3A), while the fasting plasma TG levels were unaffected between the HFru-Veh and HFru-FB group (Table 1). In agreement with an increased TG level, hepatic DAG content of the HFru-fed mice was also elevated by 53% ($p < 0.05$ vs CH) which was normalised by the treatment of FB (Figure 3B). The total content of ceramide was attenuated by 57% ($p < 0.001$ vs CH) by HFru-feeding, but was restored (ns. vs CH and CH-FB) by the treatment of FB (Figure 3C). These data suggested the implication of DAG in the apparent hepatic insulin resistance resulting from HFru feeding.

FB treatment increases hepatic fat oxidation under HFru-feeding

As enhanced FA oxidation is one of the key events resulting from the activation of PPAR α by FB in the liver (27-29), we measured molecular markers of oxidative capacity in the livers of mice. The expression of peroxisomal acyl-Coenzyme A oxidase-1 (ACOX1), a direct downstream effector of PPAR α activation, which catalyses the first step of peroxisomal β -oxidation of FAs (30), was markedly up-regulated in response to the treatment of FB (Figure 4A). Moreover, the phosphorylation of ACC which regulates the mitochondrial β -oxidation of FAs was markedly elevated (8-fold, $p < 0.001$ vs CH-Veh and HFru-Veh) in response to FB treatment in the liver of the HFru-fed mice (Figure 4B). In line with an increased oxidative capacity, the activity of β -HAD, which catalyses the third step of mitochondrial β -oxidation, was augmented by 2.4-fold ($p < 0.01$ vs CH-Veh and HFru-Veh) with FB treatment in the HFru-fed mice (Figure 4C). The activity of citrate

synthase was significantly enhanced (by 19%, $p < 0.01$ vs CH) under HFru feeding independent of PPAR α activation (Figure 4D), indicating PPAR α activation specifically enhances the oxidative capacity of the liver without affecting mitochondrial content under HFru feeding. Hepatic FA oxidation was increased (~60%) by the treatment of FB in the HFru-fed mice and this was due to an increase in the component resistant to the inhibition by etomoxir (Figure 4E).

FB treatment triggered the activation of UPR pathways in the liver

Having established that treatment of FB was effective in eliminating hepatic lipid accumulation and restoring insulin signaling, we next sought to examine its effects on the three major UPR pathways. The phosphorylation of IRE1 (Figure 5A), spliced form of XBP1 (sXBP1; Figure 5B), phosphorylation of PERK and eIF2 α (Figure 5C and D), as well as the expression CHOP (Figure 5E) were markedly enhanced by PPAR α activation regardless of the feeding conditions. In addition, the expression of GADD34, a well-characterized phosphatase of eIF2 α (9) was concomitantly down-regulated in response to PPAR α activation (Figure 5F). As expected, HFru feeding significantly increased the phosphorylated form of IRE1 (Figure 5A, $p < 0.05$ vs CH) and eIF2 α (Figure 5D, $p < 0.01$ vs CH). No changes were detected in the maturation of activating transcription factor 6 (ATF6) as a result of HFru-feeding or FB treatment (Figure S2).

FB -induced UPR signaling was accompanied by an enhanced DNL

As both activation of PPAR α (28) and UPR signaling (7) can promote DNL in the liver via the action of sterol regulatory element-binding protein-1c (SREBP1c) (31), we hence examined the expression of SREBP1c and key enzymes involve in this process. Our western blotting analysis revealed up-regulations of the mature form of SREBP1c (mSREBP1c, 3-fold, $p < 0.05$ vs CH-Veh), ACC (3.5-fold, $p < 0.01$ vs CH-Veh), FAS (2.5-fold, $p < 0.001$ vs CH) and SCD1 (14.5-fold, $p < 0.01$ vs CH-Veh) in the liver of the HFru-fed mice (Figure 6A-D). PPAR α activation in the CH-fed mice stimulated the expression of mSREBP1c, ACC, FAS and SCD1 to levels comparable to that of the

HFru-fed mice. PPAR α activation in conjunction with HFru-feeding elicited a further increase in the expression of mSREBP1c (6-fold) and SCD1 (38-fold, both $p < 0.001$ vs HFru-Veh), but not ACC (2.8-fold, $p < 0.01$ vs CH) nor FAS (2.9-fold, $p < 0.001$ vs CH, both not different vs HFru-Veh). In keeping with the up-regulated lipogenic enzymes, hepatic DNL was significantly increased (~38 %) by PPAR α activation in CH-fed mice and this increase was maintained in FB treated HFru-fed mice (Figure 6E). These data suggested the FB-induced UPR signaling may enhance the lipogenic capacity of liver independent of the effects of dietary fructose.

The downstream effects of the FB -induced UPR signaling

Production of deleterious lipids (lipotoxicity) via DNL and activation of serine/threonine kinases are key consequences of UPR signaling which interferes with insulin signal transduction at various points (15). As shown in Figure 3B, treatment of FB was able to correct the elevated DAG content induced by HFru-feeding. Meanwhile, activation of JNK and IKK are well-demonstrated consequences of UPR signaling resulting in the impairment of insulin signal transduction (3). HFru feeding did not result in a significant induction of JNK (Figure 7A) or IKK (Figure 7B), and the expression of I κ B α (Figure 7C), the downstream target of IKK (32), remained unaffected which is consistent with our previous observation (16). Despite the significant induction of the two specific arms of UPR pathways, the phosphorylation status of these kinases remained unaffected in response to FB treatment (Figure 7A to C). These data suggested PPAR α activation is effective in eliminating lipotoxicity and that the FB-induced UPR signaling did not result in the activation of these stress kinases.

DISCUSSION

The present study has established a hepatic ER stress model independent of lipid accumulation in the liver with the use of PPAR α activator fenofibrate (FB) in HFru-fed mice. This model enabled us to examine the effects of ER stress on hepatic insulin sensitivity devoid of the influence of hepatic steatosis. Our data showed that PPAR α activation completely eliminate HFru-induced hepatic steatosis and insulin resistance without altering JNK and IKK in the face of marked dual activation of the IRE1/XBP1 and PERK/eIF2 α branches of the UPR pathways. These findings indicate that hepatic steatosis, but not JNK, is required for ER stress to cause insulin resistance. To the best of our knowledge, this is the first report to demonstrate that PPAR α activation induces UPR signaling while ameliorating hepatic insulin resistance.

PPAR α is a key transcriptional regulator for lipid metabolism and it can be endogenously activated by FAs, as well as pharmacologically by agonists like FB (17). FB is a specific agonist of PPAR α commonly used to treat dyslipidaemia and hypercholesterolemia in humans (33). These beneficial effects are attributed to the PPAR α -driven peroxisomal and mitochondrial β -oxidation and microsomal ω -oxidation of FA with the liver being a major site of action (34). Our data showed that FB treatment was effective in activating PPAR α *in vivo* as evidenced by the increased expression of ACOX1 (also known as palmitoyl-CoA oxidase) (35) which is a direct target of PPAR α . The concomitant increase in VO₂ and the induction of the phosphorylated form of ACC and β -HAD activity along with the augmented FA oxidation in the liver are indicative of an enhanced oxidative capacity and energy expenditure which are consistent with the reported effects of PPAR α activation (34; 36). In line with the upregulation of ACOX1 expression, FB-induced increase in hepatic FA oxidation can be attributed to the enhanced peroxisomal oxidation that is not inhabitable by etomoxir which blocks the entry of long-chain FAs into mitochondria for oxidation. It has been reported that peroxisomal oxidation break down (very) long-chain FAs into medium and short chain FAs further oxidation in mitochondria (36). Unlike long chain FAs, the short- and medium-chain

FAs do not rely on CPT1 to enter the mitochondria (36) which may explain, at least in part, the increased oxygen consumption as observed at the whole body level. It is likely that the reduced body weight and adiposity observed only in FB treated HFru-fed mice is due to the enhanced peroxisomal oxidation which was not evident in treated CH-fed mice.

The restored HOMA-IR resulting from the lowered fasting blood glucose and insulin levels, together with the restored hepatic insulin signal transduction in the HFru-fed mice by FB are suggestive of improved insulin sensitivity in these mice. This interpretation is also supported by the striking reduction in insulin secretion in FB treated chow fed mice while maintaining unaltered glucose clearance. The insulin-sensitising effect of FB observed in the present study is consistent with our previous report of the insulin sensitizing effect of PPAR α activation in high fat fed insulin resistant rats as determined by the hyperinsulinemic-euglycemic clamp (37).

The ER plays a pivotal role in protein processing to maintain cellular homeostasis under physiological conditions through the three canonical branches of UPR signaling pathways: PERK/eIF2 α , IRE1/XBP1 and ATF6. The initialising proteins PERK, IRE and ATF6 all have sensors facing the ER lumen and they can be activated under ER stress such as the accumulation of misfolded proteins (9; 15). Activated IRE1/XBP1 pathway has been suggested to promote DNL in the liver leading to the production of lipids (4; 7). In addition, both IRE1 (10) and PERK (38) have been suggested to activate JNK and IKK. These mechanisms, acting in concert or alone, are sufficient to impair insulin signaling in the liver. However, it has been difficult to separate the effect of activated UPR pathways on hepatic insulin signaling *in vivo* from the influence of lipid accumulation. Moreover, FB is a lipid-lowering drug commonly used in humans and fructose consumption is closely related to the epidemic of obesity and fatty liver (6; 39). Thus, the approach of administration of FB to insulin resistant mice induced by HFru feeding would not only allow us to dissect such integral relationship, but also provide new insight into the mechanism relevant to

conditions in humans.

Both DAG and ceramide are key lipid intermediates linking hepatic steatosis to insulin resistance (4). Our results showed that liver DAG content was higher in HFru-fed mice (as a result of increased DNL) and this is consistent with a previous report in HFru-fed mice (46). DAG can activate protein kinases C ϵ (PKC ϵ) which in turn phosphorylates the insulin receptor substrate 1 (IRS1) at serine 307 to disrupt tyrosine phosphorylation of IRS1 (40). This could blunt IRS-mediated phosphorylation of its downstream signaling target such as Akt (41). While maintaining elevated DNL (as indicated by mSREBP1c, ACC, FAS, SCD1, and the incorporation of [³H]-H₂O into triglyceride) induced by HFru diet, FB was able to outpace DNL by a much greater effect to accelerate FA oxidation (as indicated by the 14-16% increase in VO₂, 3.9-fold, 8-fold and 2.1-fold increases in ACOX1, pACC and β -HAD, respectively), hence eliminating the accumulation of TG and DAG. This may offer an explanation towards the improved insulin signaling by FB. In HFru-fed rat, hepatic ceramide has been reported to be increased (45) and this lipid metabolite can suppress the phosphorylation of Akt via protein phosphatase 2A (42; 43). In the present study ceramide is unlikely to be a contributor for the blunted insulin signaling because its level in HFru-FB mice was similar to the level in chow-fed mice. However, the precise role of ceramide requires further investigation as the cellular location may be a key determinant of its effect on insulin sensitivity (44).

The other key mechanism for ER stress induced insulin resistance is the activation of JNK and associated stress kinases. Sustained ER stress has been shown to cause hepatic insulin resistance via the induction of JNK and IKK (8; 13) and all three canonical arms of the UPR pathways are capable of activating JNK and IKK signaling under conditions of severe ER stress (3). Consistent with our previous finding (16) HFru feeding was accompanied by the presence of ER stress. Despite further activation of the IRE1/XBP1 and PERK/eIF2 α signaling by the activation of PPAR α with FB, the

unaltered phosphorylation of JNK and IKK or I κ B α content argues against their role in the improved insulin signaling properties in the liver. In addition, cellular ceramide is also known to be implicated in the up-regulation of IKK and JNK (42). The fact that neither ceramide nor these stress-related kinases were up-regulated by FB is also consistent with our interpretation of the reduction in DAGs as a more likely mechanism for the alleviation of hepatic insulin resistance by the activation of PPAR α . Of interest, Jurczak et al. (46) has recently demonstrated alleviation of hepatic DAG accumulation in mice with conditional knockout of XBP1. The absence of XBP1 can reverse fructose-induced insulin resistance despite the presence of ER stress and JNK activation which supports the notion of DAGs being the major culprit for hepatic insulin resistance induced by HFru feeding.

It has been suggested that mild ER stress may enhance hepatic insulin signaling and protect against lipotoxicity via the induction of an adaptive UPR (47). Mice carrying liver specific deletion of IRE1 displayed overt steatosis when challenged with ER stress inducers (48), while genetic ablation of either ER stress-sensing or ER quality control molecules also resulted in the development of hepatic steatosis (49). Furthermore, IRE1 has been reported capable of repressing the expression of key metabolic transcriptional regulators, including CCAAT/enhancer-binding protein (C/EBP) β , C/EBP δ , PPAR γ , and enzymes involved in triglyceride biosynthesis (49), which suggests that UPR might be an important mechanism for mitigating steatosis. Our results in the present study highlight the need for further investigation to examine whether specific UPR signaling may in fact contribute to the PPAR α -mediated effects on insulin sensitivity.

Although attenuated body weight gain in the HFru-fed mice induced by FB may cloud our interpretation at the first glance, the pivotal role of lipids (but not the activated UPR *per se*) in ER stress associated insulin resistance is also demonstrated in CH-FB fed mice without body weight change (compared to CH-fed mice). Despite similar dual activation of both IRE1/XBP1 and

PERK/eIF2 α pathways in the FB-treated CH-fed mice, insulin-mediated phosphorylation of Akt and GSK3 β remained intact in the absence of lipid accumulation. This interpretation is consistent with a previous study showing reduced liver lipids as the underlying mechanism of improved hepatic insulin sensitivity during body weight loss in patients with type 2 diabetes (50).

In summary, our data together indicated that lipid (particularly DAG) accumulation, but not the activation of JNK or IKK, is required for ER stress to cause hepatic insulin resistance and glucose intolerance during HFru consumption. Increased peroxisomal oxidation of FAs and energy expenditure are likely to underpin the observed reduction in hepatosteatosis and insulin resistance in FB-treated HFru-fed mice despite marked increases in UPR signalling and *de novo* lipogenesis. Therefore, activation of PPAR α with FB ameliorates HFru-induced hepatic insulin resistance by eliminating lipid deposition by blocking its link with ER (Figure 8). Our findings also suggest a need for further investigation as to whether activation of specific UPR pathways may in fact contribute to the therapeutic effects of fibrate drugs which are commonly used in humans.

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GUARANTOR'S STATEMENT

Dr. Ji-Ming Ye is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

AUTHOR CONTRIBUTION STATEMENT

J.M.Y. and S.M.H.C. conceived and designed the study. S.M.H.C., R.Q.S., Z.H.C. H.W., X.Y.Z. and M.J.W. contributed to the research data. J.M.Y. and M.J.W. provided reagents, materials and analysis tools. S.M.H.C., J.M.Y., R.Q.S. and M.J.W. wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interests in this study.

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TABLE 1. Basal metabolic parameters of HFru-fed mice

	CH-Veh	CH-FB	HFru-Veh	HFru-FB
Body mass (g)				
Day 0	27.0±0.4	27.5±0.4	28.0±0.5	26.8±0.4
Day 14	28.0±0.4	27.9±0.4	29.2±0.4 ^b	22.2±0.6 ^{b,d,e}
EPI/BW	1.2±0.1	1.0±0.1	2.0±0.3 ^b	0.7±0.1 ^{a,d}
Caloric intake (Kcal/kg.day)	411±6.6	436±12.3	571±9.7 ^{b,f}	567±13.6 ^{b,f}
VO ₂ (l/kg.h)	3.23±0.07	3.70±0.14 ^a	3.50±0.03 ^a	3.78±0.13 ^{a,c,e}
RER	0.93±0.01	0.92±0.01	0.97±0.01 ^a	0.93±0.02 ^c
Blood glucose (mM)	8.4±0.3	10.5±0.4 ^b	10.2±0.5 ^b	6.6±0.4 ^{b,d}
Plasma insulin (pg/ml)	203±23	91±16 ^b	208±26	82±6 ^{d,b}
HOMA-IR	77.4±9.0	40.9±6.7 ^a	96.3±16.8	23.3±2.2 ^{b,d}
Plasma triglyceride (μM)	354±20	176±10 ^b	264±16 ^b	260±32 ^b

Male C57BL/6J male mice were fed either a CH or fructose-rich diet (HFru) for 2 weeks with or without the supplementation of a PPAR α agonist, fenofibrate (FB, 100 mg/kg/day). The data for whole body oxygen consumption (VO₂) and respiratory exchange ratio (RER) were the average values of 24 hours of measurement after 1 week of FB administration. HOMA-IR was calculated using the fasting blood glucose (mmol/L) multiplying by the fasting Insulin (mU/L) divided by 22.5. Data are means \pm SE of 8–12 mice per group. ^a $p < 0.05$, ^b $p < 0.01$ vs untreated CH-fed mice (CH-Veh); ^c $p < 0.05$, ^d $p < 0.001$ vs untreated HFru-fed mice (HFru-Veh); ^e $p < 0.001$ vs FB treated chow-fed mice (CH-FB).

FIGURE LEGENDS

Figure 1. Effects of FB treatment on glucose tolerance.

Male C57BL/6J mice were fed a high fructose (HFru) diet with or without the supplementation of fenofibrate (FB, 100 mg/kg/day) as compared to a standard laboratory chow diet (CH). The experiments were performed after two weeks of chow (CH, ●), chow with FB (CH-FB, ○), high fructose (HFru, ■) or high fructose with FB (HFru-FB, □) feeding. (A) Glucose tolerance test (GTT) was performed with an injection of glucose (2.5 g/kg, *ip*) after 5-7 hours of fasting. (B) iAUC, incremental area under the curve for blood glucose level. (C) Plasma insulin level between 30-60 min of GTT. Data are means \pm SE, 8-12 mice per group. * $p < 0.05$; †† $p < 0.01$ of the compared groups.

Figure 2. Effects of FB treatment on hepatic insulin signal transduction.

After two weeks of feeding, animals were fasted for 5-7 hours before tissue collection. Liver homogenate were prepared for immunoblotting (detail methods can be found in the supplementary information): (A) representative blots of phosphorylated- and total-Akt (Ser473) with densitometry in the liver, (B) representative blots of phosphorylated- and total- GSK3 β (Ser219) with densitometry in the liver in response to a bolus of insulin stimulation (2U/kg, *ip*). Each lane represents a single mouse. Data are mean \pm SE of 8 mice per group. All insulin stimulated groups reached statistical significance of $p < 0.01$ compared to their corresponding basal groups unless otherwise indicated. ** $p < 0.01$; ††† $p < 0.001$ of the compared groups.

Figure 3. Effects of FB treatment on hepatic lipid content.

After two weeks of feeding, animals were fasted for 5-7 hours before tissue collection. Liver homogenate were extracted for the assessment of total TG (A), DAG (B) and ceramide (C) content. Data are mean \pm SE of 8 mice per group. ** $p < 0.01$; ††† $p < 0.001$ of the compared groups.

Figure 4. Effects of FB treatment on key enzymes of FA oxidation

After two weeks of feeding, animals were fasted for 5-7 hours before tissue collection and liver homogenates were immunoblotted for key enzymes related to oxidative capacity: representative blots of ACOX1 (A), phosphorylated-ACC (Ser79) (B), the specific activities of β -HAD (C), and citrate synthase (D). Each lane represents a single mouse. Data are mean \pm SE of 10 mice per group. (E) Hepatic fatty acid (FA) oxidation was measured in separate liver homogenates using ^{14}C -palmitate as a substrate in the presence or absence of 0.02 mM etomoxir (detail methods can be found in the supplementary information). Data are mean \pm SE of 6-8 mice per group. * $p < 0.05$; ** $p < 0.01$ vs CH; † $p < 0.05$, †† $p < 0.001$ of the compared groups.

Figure 5. Effects of FB treatment on hepatic UPR signaling.

After two weeks of feeding, animals were fasted for 5-7 hours before tissue collection and liver homogenates were immunoblotted for markers of ER stress: representative blots of phosphorylated-IRE1 (Ser724) (A), spliced form of XBP1 (B), phosphorylated-PERK (Thr980) (C), phosphorylated-eIF2 α (Ser51) (D), CHOP (E), and GADD34 (F) with densitometry. Each lane represents a single mouse. Data are mean \pm SE from 8 to 10 mice per group. * $p < 0.01$ vs CH, †† $p < 0.01$ of the compared groups.

Figure 6. Effects of FB treatment on hepatic DNL.

After two weeks of feeding, animals were fasted for 5-7 hours before tissue collection and liver homogenates were immunoblotted for key enzymes related to lipogenic capacity: representative blots of the matured form of SREBP1c (mSREBP1c) (A), tACC (B), FAS (C) and SCD1 (D) with densitometry. Data are mean \pm SE of 10 mice per group. (E) Hepatic DNL was measured by the incorporation of [^3H]-H $_2\text{O}$ into hepatic triglyceride (detail methods can be found in the supplementary information). Data are mean \pm SE of 6 to 8 mice per group. * $p < 0.05$ vs CH; ** p

< 0.01; †† $p < 0.001$ of the compared groups.

Figure 7. Effects of FB treatment on JNK and IKK activation.

After two weeks of feeding, animals were fasted for 5-7 hours before tissue collection and liver homogenates were immunoblotted for evidence of JNK and IKK activation: representative blots of phosphorylated-JNK (A), phosphorylated-IKK (B), I κ B α (C). Data are mean \pm SE of 8 mice per group. n.s. denotes no statistical differences.

Figure 8. Illustration of PPAR α -mediated effects on ER stress, lipid metabolism and insulin sensitivity in the liver.

High fructose feeding accentuates the accumulation of triglyceride (TG) and diacylglycerol (DAG) in the liver via the induction of *de novo* lipogenesis. The accumulation of these lipid metabolites attenuates normal insulin signal transduction leading to hepatic insulin resistance, resulting in the reduction of glucose tolerance. PPAR α activation by FB may also directly stimulate lipogenesis which may involve the signaling of specific arms of the unfolded protein response (UPR) pathways. However, the predominant effect of potentiated oxidative capacity (primarily peroxisomal oxidation) driven by PPAR α is capable of eliminating lipid accumulation, thus overcoming fructose-induced hepatic insulin resistance (IR) and glucose intolerance.

Figure S1. Densitometry of insulin-stimulated Akt phosphorylation in muscle.

After two weeks of feeding, animals were fasted for 5-7 hours before tissue collection and red quadriceps muscle homogenates were immunoblotted for phosphorylated-Akt (Ser473) in response to a bolus of insulin stimulation (2U/kg, *ip*). Data are mean \pm SE from 8 to 10 mice per group. All insulin stimulated samples reached statistical significance of $p < 0.01$ compared to that of the non-stimulated. No significant difference was observed among the stimulated groups.

Figure S2. Effects of FB treatment on ATF6.

After two weeks of feeding, animals were fasted for 5-7 hours before tissue collection and liver homogenates were immunoblotted for ATF6. Representative blot is shown with densitometry. Each lane represents a single mouse. Data are mean \pm SE from 8 to 10 mice per group. n.s. denotes no statistical differences.